PRION BIOLOGY STUDY

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NON-TECHNICAL ABSTRACT

In the early 1980's a novel disease of cattle was first recognized. This disease, called Bovine Spongiform Encephalopathy (BSE), is a progressive neurological disorder characterized by erratic behavior and pronounced clumsiness in infected animals. Although the disease kills infected animals within a few months after symptoms appear, the appearance of symptoms can take several years. The BSE outbreak led to the destruction of thousands of herds of cattle and the collapse of the British beef industry. The US cattle industry, with over $50 billion in annual sales a year, would be ruined if BSE were diagnosed in US herds. The discovery of BSE was followed by the recognition of novel neurological diseases in several species, including mankind. Since 1996, over 100 humans in the European Union have been diagnosed with a previously undiagnosed neurological disorder, called variant Cruetzfeldt-Jakob disease (vCJD). This newly emerged disease is strikingly similar to BSE in that its victims show symptoms after a long incubation period (estimated to be approximately 10 years) and die after progressive loss of brain function. It is suspected that cattle and people became infected via eating foodstuffs contaminated with infected brain tissue.

Although there is still scientific debate about what causes BSE and vCJD, most prevailing evidence points to an entirely new class of infectious agent, called a prion, which replicates by changing the products of the cells of the host into copies of itself.

Due to the unconventional nature of prions, commonly used techniques for the diagnosis and treatment of infected patients are useless to combat prion diseases. Current tests for the diagnosis of BSE in cattle are slow, technically challenging and insensitive. There is no therapy for vCJD.

Clearly, new diagnostic tools and therapeutic regimes need to be developed to prevent the spread of vCJD or treat the victims that may be incubating the disease. In this report, recommendations are made that can guide the development of tools and drugs that would combat the spread of these newly emerged diseases. These recommendations include improvements upon existing technology and the use of technology not previously brought to bear in the battle against infectious diseases.

TECHNICAL ABSTRACT

In the early 1980's a novel disease of cattle was first diagnosed. This disease, called Bovine Spongiform Encephalopathy (BSE), is a neurological disorder characterized a long incubation time followed by progressive loss of brain function and invariably culminating in fatality. The BSE outbreak led to the destruction of thousands of herds of cattle and the collapse of the British beef industry. The discovery of BSE was followed by the emergence of novel neurological diseases in several species, including mankind. Since 1996, over 100 patients in the European Union have been diagnosed with the novel neurological disorder, strikingly similar to BSE in that its victims show symptoms after a long incubation period (estimated to be approximately 10 years) and succumb after progressive neurological decay, called variant Cruetzfeldt-Jakob disease (vCJD). It is suspected that cattle and people became infected via eating foodstuffs contaminated with infected brain tissue.

Although there is still scientific debate regarding the agent of BSE and vCJD, several lines of evidence suggest that the pathogen consists entirely of protein, called a prion. The pathogen is characterized by: extreme resistance to treatments that destroy nucleic acid, sensitivity to treatments that destroy protein, and lack of immunological response by the host. It is hypothesized that prions replicate by hijacking protein encoded by the host's genome and transmuting it into versions of itself. This transmutation, observable in vitro, involves the binding of the prion to its cellular homologue and the change of its secondary structure to resemble the prion.

Due to the fact that prions posses no nucleic acid and produce no immunological response in the host, current diagnostic techniques (such as PCR or host antibody titers) are unable to identify infected individuals. Furthermore, since the pathogen is neither a virus nor a bacterium, current therapeutics for the treatment of infectious disease are useless. Current tests for the diagnosis of BSE in cattle are slow, technically challenging and insensitive. There is no therapy for the treatment of vCJD.

Clearly, new diagnostic tools and therapeutic regimes need to be developed to prevent the spread of vCJD or treat the victims that may be incubating the disease. In this report, recommendations are made that can guide the development of tools and drugs that would combat the spread of these novel diseases.

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DEFINITIONS AND ABBREVIATIONS:

BSE: Bovine Spongiform Encephalopathy, also called Mad-cow disease.

ELISA: Enzyme-Linked ImmunoSorbent Assay, a sensitive and moderate throughput assay that uses antibodies specific to immobilize a target prior to detection.

EDRF: Erythroid Differentiation Related Factor, a blood component significantly downregulated in the BSE-infected animals

EU: European Union

Fab: A Fragment of antibodies that retains its binding affinity. Unlike the complete antibody, it is monovalent.

FFI: Fatal Familial Insomnia, an extremely rare, inherited neurological prion disease.

GSS: Gerstmann-Straussler-Scheinker Syndrome, a rare, inherited, neurological prion disease.

GPI: glycosyl phosphotidylinositol, a carbohydrate-rich molecule often serving to tether a protein to a cellular membrane.

MBM: Meat and bone meal, a feed derived from the rendered carcasses of animals that was fed to cattle.

Prion: a proteinaceous infectious particle that lacks nucleic acid.[1] More broadly, the term prion is used to describe a protein that can transmit any phenotype (not necessarily a disease) from organism to organism.

PrP-sen: wild-type, protease sensitive (sen) form of the prion protein that is not pathogenic and occurs normally in several cell types. Also called PrP^c by some authors.

PrP-res: conformer of PrP-sen that is resistant to protease treatment (res). This state is a necessary precursor to the pathogenic form of the prion, but PrP-res is not necessarily pathogenic.

PrP-sc: conformer of PrP-res that transmits disease; depending on the animal infected, this prion can cause scrapie (sheep), BSE (cows) or vCJD (humans).

TSE: Transmissible Spongiform Encephalopathy, includes BSE, CJD, GSS

UK: United Kingdom

vCJD: variant Creutzfeldt-Jakob disease, also called "human BSE".

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[1] **Pruisner, S., 1998**. Prions, **The Proceedings of the National Academy of Sciences of the USA, 95, pp. 13363-13383.**

FORWARD

This study has focused primarily on BSE and its related diseases in other species (vCJD in humans and scrapie in sheep). Although several other prion diseases unrelated in cause to BSE exist; such as fatal familial insomnia and chronic wasting disease; these diseases will only be discussed for comparison purposes and only briefly. Importantly, most of the recommendations issued by this report to bolster defense and response against the spread of BSE-related diseases will also assist the containment, diagnosis and treatment of other prion diseases.

INTRODUCTION

1. Background

1A. The Emergence of Bovine Spongiform Encephalopathy

Perhaps the greatest accomplishment of the 20th century is the advent of modern medicine. Advances in medical microbiology enabled scientists to discover the etiologic agent of many infectious diseases, which led to the discovery of vaccines to prevent disease and therapeutics to combat infection. All diseases were thought to be caused by infestation of the host with microscopic organisms (bacteria, fungi or other parasites) or viruses. Near the close of the century, we found ourselves confronted with an agricultural epidemic caused neither by an organism nor a conventional virus.

In 1986, cattle in the UK were diagnosed with a novel neurological disorder.[2] At first, the changes in the affected cattle are very subtle; the animal will prefer to stay away from the herd or seem clumsy while walking on flooring. Eventually, these symptoms increase in severity leading to hypersensitivity to sound, light and touch, and pronounced clumsiness (ataxia). In the later stages of the disease, the animals are prone to unprovoked frenzies and other erratic and violent behavior. Motor coordination declines slowly, leading to complete prostration prior to death. From the onset of the first symptoms to death is typically a few months.[3]

Upon necropsy, a pronounced destruction of brain tissue is observed. Cell death leads to vacuolization of the brain tissue causing infected tissue to resemble to a sponge. This extensive tissue damage in the host gives the disease the name by which we know it today, Bovine Spongiform Encephalopathy (BSE). When viewed microscopically, the plaques had a cleared center with zones of irregular masses of supporting cells radiating from it (gliosis), making the plaque resemble a daisy (a florid appearance). Associated with the florid plaques are bundles of tangled, amyloid fibrils, aggregates of protein. These features of the damaged tissue distinguished this disease from all other neurological ailments of cattle, confirming it as a previously uncharacterized illness.

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[2] Brown, et al., **2001.** Bovine Spongiform Encephalopathy and Variant Creutzfeldt-Jakob Disease: Background, Evolution and Current Concerns, **Emerging Infectious Diseases**, 7, 6-16.

[3] Bradley, R., **2001** Prions, a Challenge for Science, Medicine and Public Health System, **Contributions to Microbiology**, 7, pp 105-146.

The disease rapidly spread to cattle all over the UK, peaking with 35,000 infected animals in the UK in 1992. Overall, approximately 200,000 cases of BSE have been reported. The outbreak was not confined to the UK; infected cattle were found in most other EU countries, most notably Switzerland, Ireland, Portugal and France.[4] The cost of the outbreak of BSE, in terms of lost markets and eradication expenses, is estimated to be $38 billion to Europe alone up to the year 2000.[5] Wherever diseased cattle are found, consumers turn away from the purchase of beef; in Japan, where 3 cattle have been found with BSE, the consumer demand for beef has dropped by half.[6] The economic cost to the US, with beef and dairy sales exceeding $50 billion a year, would dwarf the losses seen in the EU if the disease came to this country. Worse still, spongiform encephalopathy began to appear in several zoo animals that were fed the carcasses of diseased cattle, indicating that the disease can be transmissible to unrelated species.[7]

Scientists were able to transmit BSE to other species in the laboratory by homogenizing tissue from infected cattle and injecting it into the brains of model species. This finding confirmed that BSE was a transmissible spongiform encephalopathy (TSE). It was later found that the brains and central nervous system from infected animals harbored the greatest amount of infectious material and no infectivity was found in milk, muscle, peripheral nerves or reproductive tissue.[8] One experiment has demonstrated that sheep receiving whole blood from infected sheep may contract scrapie, but the experiment is only correlative and flawed due to lack of appropriate controls.[9]

1B. The Emergence of Variant Creutzfeldt-Jakob Disease

On March 20, 1996, a new spongiform encephalopathy was found in humans. Ten new cases of Creutzfeldt-Jakob disease (CJD) was diagnosed in young Britons (mean age 26 years).[10] Although CJD is not a new disease, it is extremely rare (affecting only 1 in a

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[4] Bradley, R., **2001**. Prions, a Challenge for Science, Medicine and Public Health System, **Contributions to Microbiology**, 7, pp 105-146.
[5] Author not cited, **2001**.**Homeland Security Bulletin**, September 7.
[6] Watts, J., **2001.** Japan's Government Tried to Allay BSE Fears, **The Lancet,** 358, p 2057.
[7] Author not cited, **1986.** Report to the Chief Veterinary Officer, **Animal Health**, p69.
[8] Taylor, D. et al., **1995.** Absence of Disease in Mice Receiving Milk from Cows with Bovine Spongiform Encephalopathy, **Veterinary Records**, 136, p. 592.
[9] Houston, F. et al., **2000.** Transmission of BSE by Blood Transfusion in Sheep, **The Lancet**, 356, pp. 999-1000.
[10] Bradley, R., **2001.**

million people) and primarily affects the elderly (mean age 68 years).[11] Furthermore, the classical CJD has a much shorter pathological course than the CJD that was found in the young (mean illness duration of 4 months versus 14 months). Other clinical tests (discussed later) further distinguished the new cases of CJD as separate disease, called variant CJD or vCJD.

People afflicted with vCJD initially present with psychiatric disturbance (anxiety, agitation, depression) and phantom sensations (pain or auditory and visual hallucinations).[12] These symptoms occur in the absence of any easily observable physical abnormality.[13] Approximately six months after the first neurological symptoms appear the phantom pain increases, coordination lapses, involuntary limb movements appear and dementia ensues.[14] Rapid neurological deterioration continues until death approximately 14 months after the first symptoms appear. An excellent review of the symptoms of the first 100 patients was recently published.[15]

Today, over 100 people have been diagnosed with vCJD in the UK, Ireland and France. Little is known about the transmissibility of the disease. Estimates for the total number of currently incubating vCJD cases range from a few hundred cases to 140,000.[16,17]

In humans infected with vCJD, material derived from the central nervous system, tonsils and spleen was found to be infectious to mice (demonstrating that vCJD was also a TSE).[18] Although no infectious material can be found in blood, fears that it may be transmitted via contaminated blood caused the US to restrict blood donation from those who spent significant time in the EU.[19]

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[11] Knight, R. and Collins, S., **2001**. Prions, a Challenge for Science, Medicine and Public Health System, **Contributions to Microbiology**, 7, pp. 68-92.
[12] Will, R., **1999.** Psychiatric Features of New Variant Creutzfeldt-Jakob Disease, **Psychiatric Bulletin**, 23 pp. 264-267.
[13] Will, R. **1999.**
[14] Knight, R. and Collins, S. **2001.**
[15] Spencer, M. et al., **2002.** First Hundred Cases of Variant Creutzfeldt-Jakob Disease: Retrospective Case Note Review of Early Psychiatric and Neurological Features, **British Medical Journal,** 324, pp. 1479-1482.
[16] Cousens, S. et al., **1997.** Predicting the CJD Epidemic in Humans, **Nature**, 385, pp 197-198.
[17] Ghani, A., **2002.** The Epidemiology of Variant Creutzfeldt-Jakob Disease in Europe, **Microbes and Infection**, 4, pp. 385-393.
[18] Bruce, M. et al., **2001.** Detection of Variant Creutzfeldt-Jakob Disease Infectivity in Extraneural Tissue, **The Lancet**, 358, pp. 208-209.
[19] Casagrande, R., **2001.** Bad Blood Between the FDA and Europe, **The Scientist**, 15, p 43.

Both BSE and vCJD require long incubation times for disease to develop. If infectious material is fed to a cow, symptoms do not appear in the animal until 60 months later.[20] The first case of vCJD was diagnosed in humans in 1996, and if cases of vCJD are mapped onto the leading edge of the BSE epidemic, it is estimated that the incubation time in the most susceptible human hosts is approximately 10 years.

1C. Origin of BSE and vCJD

Although the temporal similarity between the BSE and vCJD outbreaks suggested a common pathogen, little was known about how either disease was transmitted. Knowledge gained from the study of a similar disease of sheep, scrapie, helped uncover how BSE could be transmitted from cow to cow and from cattle to humans. Scrapie, a progressive, fatal, neurological disease of sheep and goats, has been known for approximately 200 years and occurs in almost every country. The brains of afflicted sheep display similar pathophysiolgy to that of the brains of cattle afflicted with BSE. Sheep affected by scrapie present with the similar symptoms (ataxia, unpredictable behavior) to cattle infected with BSE. Both agents can be transmitted by injecting infectious material into the brains of other animals after a long incubation period.[21]

There are some differences between scrapie and BSE. Although sheep afflicted with scrapie have been found in almost every country, there is no history of increased neurological diseases in people or other animals where scrapie is more prevalent.[22] Furthermore, scrapie is primarily transmitted via contact: the presence of infected animals in a herd leads to the infection of more infected animals in the same herd over time.[23] In contrast, BSE does not spread amongst the members of a herd except rarely from cow to calf.

If BSE is not transmitted directly from animal to animal, how does it spread? In 1987, the first epidemiological survey was taken to determine how BSE was transmitted

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[20] Dawson, M. et al., **1990**. Preliminary Evidence of the Experimental Transmissibility of Bovine Spongiform Encephalopathy to Cattle, **Veterinary Records**, 126, pp 112-113.
[21] Fraser, H. et al., **1988**. Transmission of Bovine Spongiform Encephalopathy to Mice, **Veterinary Records**, 123, p. 472.
[22] Zerr, I. et al., **1979**. Creutzfeldt-Jakob Disease: Patterns of Worldwide Occurrence and the Significance of Familial and Sporadic Clustering, **Annals of Neurology**, 5, pp. 177-188.
[23] Hoinville, L., **1996**. A Review of the Epidemiology of Scrapie in Sheep, **Revue scientifique et technique of the Office International des Epizooties**

and if there was a link between scrapie and BSE.[24] It was found that there was no link between the presence of sheep on a farm and the incidence of BSE, therefore scrapie was not spread directly to cows from sheep. Furthermore, no link was found between the use of pesticides, fertilizers, household or industrial chemicals, vaccines or hormones to BSE incidence. The only link was that all infected cattle had been given feed containing the rendered carcasses of other cattle and sheep (meat and bone meal—MBM—based feed).[25] Locations that had a higher concentration of MBM in their feed had a higher prevalence of BSE.[26] However, MBM has been an ingredient of cattle feed for several decades before the outbreak, but BSE only was recognized in the 1980's. The reason for this late appearance has been blamed on a change in the processing of MBM. In the late 1970's and early 1980's rendering plants began to decrease the use of hydrocarbon solvents in the processing of MBM due to safety concerns. It is hypothesized that the hydrocarbon solvents used previously in the production of MBM destroyed the infectious agent and the removal of solvents from the process allowed the infectious agent to be carried in the feed. The cessation of solvent extraction of MBM allowed enough infectious agent to appear in cattle feed to cause an epidemic through the feed. Furthermore, the epidemic spread as it did because animals infected with BSE from the feed were themselves rendered and contaminated more and more cattle feed in turn.[27] This hypothesis is reinforced by the fact that the epidemic subsided after animal-derived feed was banned from being given to cattle. BSE infection in cattle born after the feed ban is attributed to cattle being given feed obtained before the feed ban.[28]

It was subsequently hypothesized that the infectious agent originally came from scrapie infected sheep. The change in feed processing allowed the scrapie-agent from being included in an infectious state in the feed of cattle. Researchers believe that the UK was the country primarily affected by BSE because sheep outnumber cattle in the UK by about 4:1 (higher than any other country in which scrapie exists), allowing scrapie-

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[24] Wilesmith, J. et al., **1988**. Bovine Spongiform Encephalopathy: Epidemiological Studies, Veterinary **Records**, 123, pp. 638-644.
[25] Wilesmith, J. et al, **1988**.
[26] Ibid.
[27] **Bradley, R., 2001**.
[28] Ibid.

infected material to reach a higher percentage of MBM than in any other country.[29] Alternatively, the strain of scrapie that predominates in the UK could be uniquely suited for transmission to cattle. This is reinforced by the fact that scrapie-infected material derived from sheep in the US cannot infect cattle with a BSE-like illness.[30]

The link of BSE to vCJD was more tenuous. No commonality could be found amongst vCJD patients in occupation, diet or medical history.[31] MBM is not a known component of the human diet. However, the proven transmission of BSE to cattle via feed and lack of consumer confidence in beef prompted the government to initiate the Specified Bovine Offal Ban which attempted to ban practices that allowed bovine nervous tissue to be included in food meant for human consumption. Firmer evidence for the transmission of BSE to people had to wait for molecular studies on the nature of the BSE agent.

Table 1. Comparative characteristics of BSE and vCJD

Disease	Characterisitics
BSE	Progressive ataxia, irritability, invariably fatal, spongiform changes in brain, florid plaques Occurs primarily in the UK or in cattle exported from the UK Devastating to beef and dairy markets where found Associated with consumption of MBM
vCJD	Progressive ataxia, irritability, invariably fatal, spongiform changes in brain, florid plaques Occurrs primarily in the UK No known risk factors

2. The Biology of Prion Diseases

2A. Prion Theory

The method of transmission from sheep to cows may have been elucidated but the nature of the agent itself remains a matter of controversy. Because of its longer history, most studies on TSEs were begun on scrapie prior to the emergence of BSE. The first indication of the unusual nature of the etiologic agent of scrapie came from treatment of infectious samples with ionizing and UV radiation.[32] Samples from scrapie infected sheep retain their virulence even after treatment with radiation that would destroy the genome

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[29] Ibid.
[30] Cutlip, R., et al., **1994**. Intracerebral Transmission of Scrapie to Cattle. **The Journal of Infectious Disease**, 169, pp. 814-820.
[31] London Department of Health, **1998.** CJD Report, **Creutzfeldt-Jakob Disease in the UK**, p. 51.
[32] Alper, T. et al., **1966.** The Exceptionally Small Size of the Scrapie Agent, **Biochemical and Biophysical Research Communications**, 22, pp. 278-284.

of any known bacteria or virus. Furthermore, unlike bacteria or viruses, no immune response is mounted by infected animals against the pathogen.[33] Also, no microorganisms or virus particles could be visualized by electron-microscopy in samples of the infectious material.[34] Later experiments demonstrated that there were too few nucleic acid pieces of sufficient length that could support virus replication in a sample to account for the number of infectious units in a sample.[35] The first hypothesis that the infectious agent could be a self-replicating protein (more resistant to radiation due to its chemical nature and small size) came in 1967.[36]

This hypothesis remained largely unsupported until 1982 when a systematic study demonstrated that chemical and physical treatments that destroy nucleic acids have no effect on infectivity whereas treatments that destroy protein deactivate the agent.[37] Prusiner, who later won the nobel prize for his work, called these proteinaceous infectious particles prions.[38] Despite this evidence, several scientists still maintain that the infectious agent is a tiny virus (virino) with a radiation-resistant genome that has escaped detection (see The Case for an Associated Virus).

Once it was determined that the infectious agent was very likely a protein, the next step was to identify that protein. The lesions in the brains of infected animals (in scrapie, BSE and vCJD) often have associated amyloid fibrils. The main component of these fibrils is a protein called the prion protein (PrP), and isolated fibrils consisting of PrP isolated from infected animals are able to pass scrapie on to other animals.[39] Through the use of reverse genetics (using the sequence of the isolated protein to deduce the sequence of the encoding gene), the gene encoding PrP was found. The Prp gene exists in single copy in the genomes of cattle, sheep and humans and the PrP protein is

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[33] Kaaden, O., **2001**. Prions, a Challenge for Science, Medicine and Public Health System, **Contributions to Microbiology**, 7, pp. 145-150.
[34] Riesner, D., **2001**. Prions, a Challenge for Science, Medicine and Public Health System, **Contributions to Microbiology**, 7, pp. 7-20.
[35] Kellings, K., et al., **1994.** Nucleic Acids in Prion Preparations: Unspecific Background or Essential Component?, **Philosophical Transactions of the Royal Society of London: Series B: Biological Sciences**, 343, pp.425-430.
[36] Griffith, J., **1967**. Self-replication and Scrapie, **Nature**, 215, pp. 1043-1044.
[37] Pruisner, S. **1982.** Novel Proteinaceous Infectious Particles Cause Scrapie, **Science**, 216, pp. 136-144.
[38] Pruisner, S., **1998**. Prions, **The Proceedings of the National Academy of Sciences of the USA**, 95, pp. 13363-13383.
[39] Prusiner, S., **1989**. Scrapie Prions, **Annual Reviews of Microbiology**, 43, pp. 345-374.

present in uninfected tissue.[40] The PrP found in healthy tissue has the same amino acid sequence as PrP isolated from diseased tissue.[41] Researchers wondered how non-diseased tissue can express PrP and remain unaffected when the presence of the PrP from diseased tissue is linked to pathology. The first difference between the normal and pathogenic PrP found was their differential resistance to proteolysis; PrP isolated from diseased tissue is resistant to enzymes that destroy protein whereas PrP isolated from healthy tissue is sensitive to such treatment.[42] Furthermore, if pathogenic PrP is denatured or solubilized in detergent it loses its infectivity.[43] The protease resistance of pathogenic PrP is found to be at least partially due to its tendency to aggregate into fibrils as solubilization in detergents restore sensitivity to the proteolytic enzymes.[44] Researchers reasoned that this difference leads to the pathogenic properties of the PrP derived from scrapie-infected animals, PrP-sc.

In order for a disease to be infectious, the pathogen must be able to replicate, otherwise the pathogen would be diluted in each new animals it infects, eventually losing pathogenicity. The first hint of how PrP-sc replicates came from experiments in which PrP from uninfected tissue, PrP-sen, was mixed with PrP-sc. When proteins from both sources are incubated together, PrP-sen is converted into a protease-resistant form, PrP-res that is structurally very similar to PrP-sc.[45] Also, mice lacking the Prp gene (and thus do not make any PrP-sen) are completely resistant to infection by PrP-sc and tissue taken from these mice after inoculation with PrP-sc does not lead to infection in wild-type animals.[46]

These findings led Stanley Prusiner to elaborate the theory on prion replication (figure 1), which lead to his receiving the Nobel Prize. As PrP-sc enters the cell, it interacts with PrP-sen synthesized by the cell. This interaction causes PrP-sen to be

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[40] Oesch, B. et al., **1985.** A Cellular Gene Encodes Scrapie PrP 27-30 Protein, **Cell**, 40, pp. 735-746.
[41] Stahl, N. et al., **1983.** Structural Analysis of the Scrapie Prion Protein Using Mass Spectrometry and Amino Acid Sequencing, **Biochemistry**, 32, pp. 1991-2002.
[42] McKinley, M., et al., **1983.** A Protease-resistant Protein is a Structural Component of the Scrapie Prion, **Cell**, 35, pp. 57-62.
[43] Prusiner, S., et al., **1993.** Attempts to Restore Scrapie Prion Infectivity after Exposure to Protein Denaturants, **The Proceedings of the National Academy of Sciences of the USA**, 90, pp. 608-621.
[44] Prusiner, S., et al., **1993.**
[45] Caughey, B. et al., **1991.** Secondary Structure Analysis of the Scrapie-associated Protein PrP 27-30 in Water by Infrared Spectroscopy, **Biochemistry**, 30, pp. 7672-7680.
[46] Buehler, H. et al., **1983.** Mice Devoid of PrP are Resistant to Scrapie, **Cell**, 356, pp. 1339-1347.

converted into PrP-sc, which accumulates due to its protease resistance. As the cell produces more PrP-sen, it is converted into PrP-sc, magnifying the pathogen.[47]

From this model, one would predict that the PrP-sc derived from cattle would primarily have the amino acid sequence of cattle PrP-sen; PrP-sc derived from sheep would have the sequence corresponding for sheep. Therefore, the PrP-sc collected from infected cattle should interact more efficiently than sheep PrP-sen with the PrP-sen in cattle due to its similarity, facilitating pathogenicity. In fact, cattle (and all other animals tested) develop disease much more rapidly when inoculated with PrP-sc derived from the same species. PrP-sc obtained from other species infects its host less efficiently in time and amount, a phenomenon known as the species barrier.[48] After passage though an animal of the same species, however, the infectious agent becomes as infectious as if it were originally derived from the same species. For example, if a mouse is injected with cattle PrP-sc, it would develop disease somewhat slowly. After this mouse falls ill, PrP-sc derived from the ill mouse can then efficiently infect other mice (due to the fact that the PrP-sc is primarily derived from mouse PrP-sen).[49] In fact, transgenic mice with the bovine Prp gene instead of the mouse version of the gene are infected by bovine PrP-sc with equal efficiency as cattle.[50] The species barrier functions also in vitro: when PrP-sc from one animal is mixed with PrP-sen from another, the conversion of PrP-sen into a PrP-sc like state is inefficient if the animals are not closely related (like sheep and mankind).[51]

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[47] Pruisner, S., **1998**.

[48] Ibid.

[49] Ibid.

[50] Prusiner, S. et al., **1990.** Transgenic Studies Implicate Interactions Between Homologous PrP Isoforms in Scrapie Prion Replication, **Cell**, 63, pp. 673-686.

[51] Raymond, G. et al., **2000.** Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease, **The EMBO Journal**, 19, pp. 4425-4430.

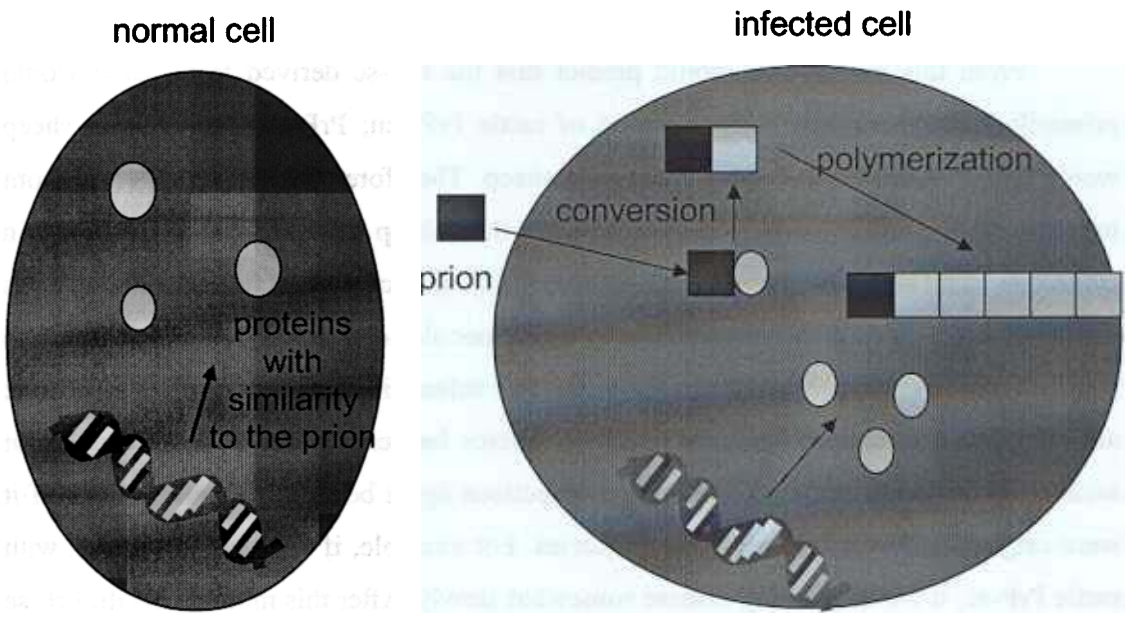

Figure 1. Cartoon illustrating prion propagation. Adapted from Pruisner, S., 1998.

Genetic variation *within* the human population seems to affect the susceptibility to vCJD. The human prion protein can have either valine or methionine for the amino acid in position 129. All patients with vCJD to date are homozygous for the Prp allele that encodes methionine at position 129 (approximately 40% of the population).[52] The bovine Prp gene also encodes methionine at position 129, suggesting that this similarity may reduce the species barrier between cattle and humans. However, it is unknown whether people that have other Prp genotypes are completely immune to vCJD or simply will have a longer incubation time of the disease.

2B. The Link Between vCJD and BSE

The emergence of new TSEs in over ten species that feed on cattle (including mankind) since the appearance of the BSE epidemic suggested a causal link between BSE and vCJD. Also, mice that only express the human PrP-sen can be infected with

[52] Knight, R. and Collins, S. **2001.** Prions, a Challenge for Science, Medicine and Public Health System, **Contributions to Microbiology**, 7, pp. 68-92.

BSE, indicating that humans could be susceptible to infection by BSE.[53] It was assumed that people could get infected via bovine neural tissue that accidentally was incorporated into human food.

More rigorous evidence came with molecular studies. The PrP-sc harvested from patients with vCJD, cattle with BSE, or zoo animals infected with various spongiform encephalopathies associated with BSE all produce identical pathology in mice (in contrast to PrP-sc harvested from classical CJD patients), indicating that they are the same strain.[54]

PrP-sc found associated with various prion diseases (like familial fatal insomnia, discussed in Other Prion Diseases, below) all show slightly different biochemical properties (migration on analytical gels or glycosylation pattern). However, PrP-sc harvested from the brains of patients who died of vCJD show identical biochemical properties to PrP-sc harvested from cattle and distinct properties from PrP-sc harvested from patients who died of classical CJD.[55] These biochemical differences in the biochemical properties of PrP-sc are now used as a differential diagnosis between vCJD and CJD.

2C. Cell Biology of the Prion Protein

Once it was proven that scrapie progression requires the synthesis of the prion protein by the host, experiments were undertaken to determine the role of PrP-sen in the cell. Transcription off the Prp gene leads to production of Prp mRNA is most neural tissues. The levels of Prp mRNA fluctuate in response to the time of day (circadian regulation) although this finding has unknown significance.[56] The level of most proteins is regulated by controlling the transcription off their encoding gene. The amount of PrP protein, however, seems to be largely controlled after transcription as significant amounts

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[53] Collindge, J. et al., **1995.** Unaltered Susceptibility to BSE in Transgenic Mice Expressing Human Prion Protein, **Nature**, 378, pp. 779-783.
[54] Bruce, M. et al., **1997.** Transmission to Mice Indicates that New Variant CJD is caused by the BSE agent, **Nature**, 389, pp. 498-501.
[55] Collinge, J. et al., **1996.** Molecular Analysis of Prion Strain Variation and the Aetiology of New Variant CJD, **Nature**, 383, pp. 685-690.
[56] Cagampang, F. et al., **1999.** Circadian Regulation of Prion Protein Messenger NRA in the Rat Forebrain: a Widespread and Synchronous Rhythm, **Neuroscience**, 91, pp. 1201-1204.

of Prp mRNA can be detected in cells that express no PrP protein.[57] It is possible that this post-transcriptional regulation is due to alternative adenylation of the Prp mRNA by an unknown enzyme.[58]

Translation of the Prp mRNA leads to the synthesis of a 250 amino acid protein that contains an N-terminal signal peptide to guide its insertion into the secretory pathway. Concurrent with translation, PrP-sen is inserted into the endoplasmic reticulum where the signal peptide is removed, a disulphide bridge is formed and up to two N-linked glycans are added.[59] Also in the endoplasmic reticulum, the C-terminus of the protein is removed and a glycosyl phosphotidylinositol (GPI) anchor is added to tether the nascent protein to the membrane.[60] Most GPI-linked proteins with N-terminal signals are completely inserted into the lumen of the endoplasmic reticulum, however experimental evidence suggests that there is an alternative form of the PrP protein can exist as an integral membrane protein with its N-terminus in the cytoplasm.[61] The significance of this alternate orientation remains unclear.

From a combination of structural analyses including nuclear magnetic resonance analysis and studies measuring the circular dichroism of PrP in solution, it was determined that the secondary structure (folding conformation) of PrP-sen differs greatly from that of PrP-sc (figure 2).[62,63] PrP-sen folds primarily through the organization of α-helices whereas PrP-sc adopted largely β-sheet content. Furthermore, the co-incubation of PrP-sen with PrP-sc caused PrP-sen to change secondary structure and adopt a form dominated by β-sheet content.[64] Due to difficulties associated with solving the structure of insoluble and membrane proteins, a complete X-ray crystallographic structure that

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[57] Ford, M. et al., **2002.** A Marked Disparity Between the Expression of Prion Protein and Its Message by Neurones of the CNS, **Neuroscience**, 111, pp. 533-551.
[58] Goldmann, W. et al., **1999.** PrP (Prion) Gene Expression in Sheep May be Modulated by Alternative Polyadenylation of Its Messenger RNA, **The Journal of General Virology**, 80, pp.2275-2283.
[59] Caughey, B. et al., **1989.** Prion Protein Biosynthesis in Scrapie-infected and Uninfected Neuroblastoma Cells, **The Journal of Virology**, 63, pp. 175-181.
[60] Stahl, N. et al., **1992.** Glycosylinositol Phospholipid Anchors of the Scrapie and Cellular Prion Proteins Contain Sialic Acid, **Biochemistry**, 31, pp. 5043-5053.
[61] Hedge, R. et al., **1998.** A Transmembrane Form of the Prion Protein in Neurodegenerative Disease, **Science**, 279, pp. 827-834.
[62] Huang, Z. et al., **1994.** Proposed Three-dimensional Structure for the Cellular Prion Protein, **The Proceedings of the National Academy of Sciences of the USA**, 91, pp. 7139-7143.
[63] Pan, K. et al., **1993.** Conversion of α–helices into β-sheets Features in the Formation of the Scrapie Prion Proteins, **The Proceedings of the National Academy of Sciences of the USA**, 90, pp. 10962-10966.
[64] Pan, K. et al., **1993.**

locates each atom in space is unavailable. Furthermore, the N-terminus of PrP-sen is highly flexible, preventing structural analysis.

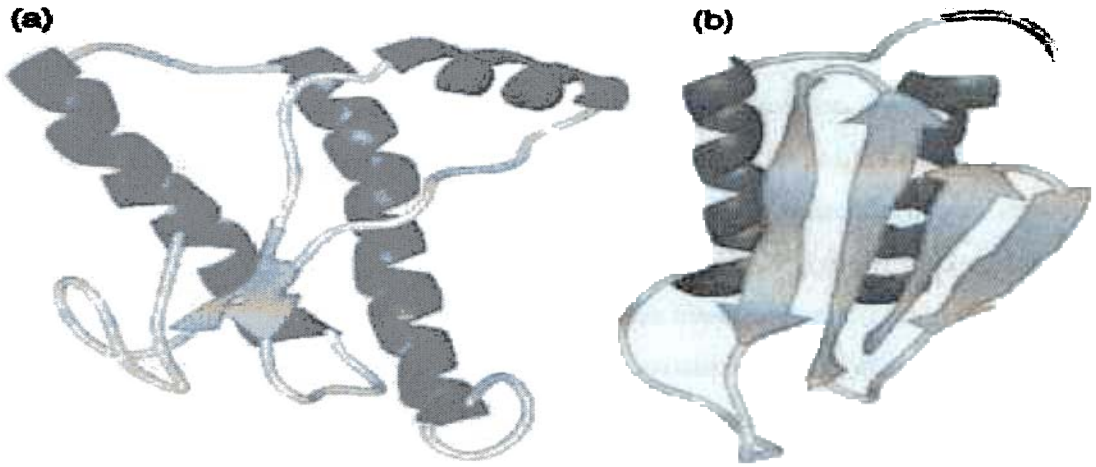

Figure 2. Ribbon diagrams depicting the secondary structure of PrP-sen (a) and PrP-sc (b); adapted from Cohen, F., 1999, Protein Misfolding and Prion Diseases, The Journal of Molecular Biology, 293, pp. 313-320. Arrows depict β-strands and helices depict α-helices.

After folding in the endoplasmic reticulum, the protein travels through the Golgi apparatus to the cell surface. It is at the cell surface where the majority of mature PrP-sen can be found, bound to the cell via its GPI anchor. On the cell surface of neurons, PrP-sen is primarily found in the synaptic region, although the mechanism of this homing is unknown.[65]

PrP-sen continuously cycles between its cell-surface location and an endocytic compartment, in a manner similar to cell surface receptors. In this endocytic compartment, a protease cleaves off the N-terminal quarter of the protein before the remaining protein is delivered back to the cell surface.[66] The endocytosis of PrP-sen into the cell occurs after concentration of the protein in special membranous structures called clathrin-coated pits.[67] GPI anchored proteins are not able to associate into clathrin-coated pits by themselves and require a receptor for their localization. Therefore, it is

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[65] Borchelt, D. et al., **1994.** Rapid Anterograde Axonal Transport of the Cellular Prion Glycoprotein in the Peripheral and Central Nervous Systems, **The Journal of Biological Chemistry**, 269, pp. 14711-14714.
[66] Shyng, S. et al., **1993.** A Prion Protein Cycles Between the Cell Surface and an Endocytic Compartment in Cultured Neuroblastoma Cells, **The Journal of Biological Chemistry**, 268, pp. 14793-14800.
[67] Anderson, R. **1993**. Each Caveola Contains Multiple Glycosyl-phosphatidylinositol-anchored Membrane Proteins, **Current Opinion in Cell Biology**, 5, pp. 647-652.

hypothesized that PrP-sen cycling to an endocytic compartment, and perhaps its cellular function, requires the action of an as yet unknown PrP receptor protein.[68] PrP-sen can also be removed from the cell surface via the action of extra-cellular enzymes that cleave the GPI anchor, releasing PrP-sen into the extra-cellular space.

The function of PrP-sen in the cell remains unclear. Its cellular location suggests a role as a receptor or adhesion molecule. In fact, when PrP-sen is over-expressed on the cell surface, cells tend to clump more tenaciously, reinforcing the possibility that PrP-sen functions in cell adhesion.[69] Concerning the role of PrP-sen as a receptor, PrP-sen has been observed to strongly bind only copper ions (femtomolar affinity).[70] In vitro, PrP-sen (with copper bound) has significant superoxide dismutase activity, indicating that PrP-sen may protect the cell against oxidative stress.[71] Interestingly, PrP-sc cannot bind copper, binding zinc and manganese preferentially, and lacks dismutase activity.[72] Also, antibodies against PrP-c block neuron differentiation through laminin signaling, suggesting a role of PrP-c in the development of the brain.[73] Another theory has cellular PrP working as a neuronal receptor that prevents neuronal cell death when engaged by Stress-inducible Protein 1.[74,75]

Despite these activities attributed to PrP-sen, mice lacking the Prp gene (and therefore have no PrP-sen) are seemingly normal.[76] Neurological defects, including neural degeneration and ataxia, are observed if the Prp gene is ablated in a way that affects the transcription of a closely related gene, doppel.[77] It is hypothesized that the

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[68] Harrris, D. et al., **1996.** Cell Biology of the Prion Protein, **Current Topics in Microbiology and Immunology**, 207, pp. 77-93.
[69] Mange, A. et al., **2002.** PrP-dependant Cell Adhesion in N2a Neuroblastoma Cells, **FEBS Letters,** 514, pp. 159-162.
[70] Hornshaw, M. et al., **1995.** Copper Binding to the N-terminal Tandem Repeat Regions of Mammalian and Avian Prion Protein, **Biochemical and Biophysical Research Communications**, 214, pp. 993-999.
[71] Brown, D. et al., **1999.** Normal Prion Protein Has an Activity Like that of Superoxide Dismutase, **The Biochemical Journal**, 344, pp. 1-5.
[72] Wong, B. et al., **2001.** Aberrant Metal Binding by Prion Protein in Human Prion Disease, **The Journal of Neurochemistry**, 78, pp. 1400-1408.
[73] Graner, E. et al., **2000.** Cellular Prion Protein Binds Laminin and Mediates Neuritogenesis, **Molecular Brain Research**, 76, pp. 85-92.
[74] Chiarini, L. et al., **2002.** Cellular Prion Protein Transduces Neuroprotective Signals, **The EMBO Journal**, 21, pp. 3317-3326.
[75] Zanata, S. et al., **2002.** Stress-inducible Protein 1 is a Cell Surface Ligand for Cellular Prion that Triggers Neuroprotection, **The EMBO Journal**, 21, pp. 3307-3316.
[76] Bueller, H. et al., **1992.**
[77] Moore, R. et al., **1999.** Ataxia in Prion Protein (PrP)-deficient Mice is Associated with Upregulation of the Novel PrP-like Protein Doppel, **The Journal of Molecular Biology**, 292, pp. 797-817.

doppel protein can compensate for the loss of the structurally similar PrP-sen but not be converted into pathogenic prions by PrP-sc.[78] If the Prp gene is knocked-out in adult mice (that previously had normal levels of PrP), subtle defects in mitochondrial structure are observed.[79]

2D. Physiology of the Uptake of PrP-sc

The interaction of PrP-sc with the cell can be easily observed in vitro but the path that PrP-sc takes when ingested by an animal is still somewhat murky. Prior to accumulation in the brain, PrP-sc is first detected in the lymphoreticular system (including the spleen and tonsils).[80] White blood cells (leukocytes) that travel through the blood stream and then migrate back to the lymphoreticular organs, were also found to accumulate significant amounts of PrP-sc in infected animals.[81] Treatments that block the differentiation or homing of leukocytes significantly retard the progression of scrapie.[82,83,84] It is hypothesized that leukocytes scavenge PrP-sc from the blood and then return to the organs of the lymphoreticular system. Infected leukocytes are then suspected of carrying PrP-sc to the brain via sympathetic nerves that innervate the lymphoreticular system because strains of mice that have greater innervation of these tissues have a reduced incubation time of the disease.[85] Exactly how or why the white blood cells carry PrP-sc to the brain remains a mystery.

It is still unclear how PrP-sc accomplishes its pathogenic effects on neurons in the brain. Increases in neuronal apoptosis (programmed cell death) and oxidative damage to

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[78] Moore, R. et al., **1999.**

[79] Miele, G. et al., **2002.** Ablation of Cellular Prion Protein Expression Affects Mitochondrial Numbers and Morphology, **Biochemical and Biophysical Research Communications,** 291, pp. 372-377.

[80] Hill, A. et al., **1999.** Investigation of Variant Creutzfeldt-Jakob Disease and Other Prion Diseases with Tonsil Biopsy Samples, **The Lancet**, 353, pp. 183-189.

[81] Brown, K. et al., **1999**. Scrapie Replication in Lymphoid Tissues Depends on PrP-expressing Follicular Dendritic Cells, **Nature Medicine**, 5, pp. 1308-1312.

[82] Mabbot, N. et al., **2000**. Temporary Inactivation of Follicular Dendritic Cells Delays Neuroinvasion of Scrapie, **Nature Medicine**, 6, pp. 719-720.

[83] Oldstone, M. et al., **2002.** Lymphotoxin-a- and Lymphotoxin-b-deficient Mice Differ in Susceptibility to Scrapie: Evidence Against Dendritic Cell Involvement in Neuroinvasion, **The Journal of Virology**, 76, pp. 4357-4363.

[84] Manuelidis, L. et al., **2000**. Follicular Dendritic Cells and Dissemination of Creutzfeldt-Jakob Disease, **The Journal of Virology**, 74, pp. 8614-8622.

[85] Glatzel, M. et al., **2001.** Sympathetic innervation of lymphoreticular organs is rate limiting for prion neuroinvasion, **Neuron**, 31, pp. 25-34.

DNA in neurons are observed as PrP-sc accumulates, however it is uncertain if these effects are due to the action of PrP-sc, the absence of PrP-c function, or just symptomatic of neural pathology.[86,87] Recent evidence suggests that PrP-sc exerts its effects through a receptor on the surface of neurons, neurotrophin p75 receptor, as cells lacking this receptor seem to be resistant to damage caused by fragments of PrP-sc.[88]

Table 2. Cell biology of the prion protein

Characteristics of the infectious agent	Resistance to treatments that destroy nucleic acid, sensitivity to treatments that destroy protein No observable nucleic acid of sufficient length to support a virus
Characteristics of PrP-sc	Identical amino acid sequence to a cellular protein Largest constituent of the agent of BSE Folds into a protease-resistant, aggregation-prone, β-sheet form PrP-sc isolated from humans or cattle has identical modifications Can convert PrP-c into a PrP-sc-like form
Characteristics of PrP-c	Folds into an α-helical-dominated form, plasma membrane protein, Protease sensitive No tendency to aggregate Possibly functions as a receptor in the brain, binds copper, has superoxide-dismutase activity
Theoretical method of uptake of PrP-sc by the body	Ingestion is followed by concentration in leukocytes that surveil the gut, Infected lymphocytes travel to the brain via nerve cells that innervate the gut

3. The Case for an Associated Virus

Despite several lines of evidence that suggest that BSE and vCJD are caused by PrP-sc, some researchers maintain that BSE and vCJD are caused by a virus that remains elusive to this day. This virus has to have several unusual characteristics: it must not elicit and immune response in its hosts, it must be extremely small or masked by host protein to avoid detection by electron microscopy and it must be extremely resistant to treatments that destroy nucleic acid. Although it seems unlikely that a virus could have any or all of these characteristics, these researchers point out some gaps in prion theory.

One characteristic originally thought to be in contrast with prion theory is the existence of prion strains. In brief, prions taken from different sources can produce different symptoms in host animals (such as lesion patterns and distribution of damage to

[86] Guentchev, M. et al., **2002.** Oxidative Damage to Nucleic Acids in Human Prion Disease, **Neurobiological Disorders**, 9, pp 275-281.
[87] Siso, S. et al., **2002.** Abnormal Synaptic Protein Expression and Cell Death in Murine Scrapie, **Acta Neuropathol**, 103, pp. 615-626.
[88] Della-Bianca, V. et al., **2001.** Neurotrophin p75 Receptor is Involved in Neuronal Damage by Prion Peptide 106-126.

the brain or incubation time). Even after passage in genetically identical animals, when the PrP-sc produced should be identical because it is encoded by identical genes, these strains still produce distinct patterns.[89] Studies with transmissible mink encephalopathy led to the elucidation of how "identical" proteins could lead to distinct strains. There are two strains of the mink disease, causing the afflicted animals to behave in a "hyper" or "drowsy" manner. Even though the Prp gene in all farmed mink is identical, when PrP-sc is harvested from these animals, the two strains of PrP-sc have different extents of carbohydrate modification.[90] This study with mink encephalopathy showed that prion strains adopt subtly different shapes which alter the extent of carbohydrate modifications and, in turn, alter subtle characteristics of the disease. This experiment led to the finding that PrP-sc from BSE infected cattle is modified biochemically in an identical manner to that from patients with vCJD (mentioned above), indicating that the same agent probably causes both diseases. Instead of dismantling the theory, the observation of prion strains helped buttress the theory against its critics.

Two other shortcomings of prion theory have yet to be addressed. One set of researchers could observe no PrP-sc in mice infected with BSE, even though the mice died of a BSE-like disease and could pass the disease onto other mice. As the disease was passed to more and more mice, PrP-sc was observed.[91] These scientists concluded that PrP has nothing to do with virulence, but is simply associated with the disease once the agent becomes adapted to the new host. Also, in humans or mice afflicted by FFI, PrP-sc detection is also difficult.[92,93] There have been no serious refutations of the observations cited in this paper.

The ultimate proof of prion theory would be to produce synthetic PrP-sc that causes disease in animals. Researchers have been able to produce PrP protein that folds into a PrP-sc-like form, that is resistant to proteases and converts PrP-sen. However, this

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[89] Kimberlin, R. et al., **1987.** Temporary and Permanent Modifications to a Single Strain of Mouse Scrapie in Transmission to Rats and Hamsters, **The Journal of General Virology**, 68, pp. 1875-1881.
[90] Bessen, R. et al., **1995.** Non-genetic Propagnation of Strain-specific Properties of Scrapie Prion Protein, **Nature**, 375, pp. 698-700.
[91] Lasmezas, C. et al., **1997.** Transmission of the BSE Agent to Mice in the Absence of Detectable Abnormal Prion Protein, **Science**, 274, pp. 402-404.
[92] Medori, R. et al., **1992.**
[93] Collinge, J. et al., **1995.** Transmission of Fatal Familial Insomnia to Laboratory Animals, **The Lancet**, 346, pp. 569-570.

synthetic protein is unable to produce an infectious disease in host animals, indicating that structural similarity to PrP-sc is insufficient to cause pathogenicity. The closest approach to date of a proof of prion theory has been afforded by experiments with the fungus *Podospora anserina*.[94] Prions in this organism cause death of the organism after mating with incompatible strains of the fungus. *P. anserina* prions made in vitro with a peptide synthesizer are able to produce identical phenomena when introduced into an otherwise healthy strain.[95] This is the first, and to date only, experiment where synthetic prions can cause a disease-like state in its host.

At the present time, there are no known agents that are more likely to cause vCJD and BSE than PrP-sc. Even if PrP-sc is not the causative agent of these diseases, its association with neuropathology is the best diagnostic tool we currently have to detect infection.

Table 3. Evidence for and against prions as the infectious agent of BSE and vCJD

Evidence that Supports Prions as the Infectious Agent	Evidence that Supports a Virus as the Infectious Agent
No detectable nucleic acid of sufficient length or concentration to support virus replication in infectious samples Sensitivity to treatments that destroy protein Unfolding of PrP-sc in infectious material destroys infectivity Strain-differences in prion diseases can be traced to subtle differences in post-translational prion modification Disease can be caused by synthetic prions in fungi Mice that over-express PrP-c are hyper-sensitive to infection Mice that lack PrP-c are resistant to infection	Absence of detectable PrP-sc in some mice that died of BSE Transmissibility of BSE in some mice in the absence of detectable PrP-sc Inability to synthetically produce PrP-sc that causes disease in animals

4. Other Prion Diseases

4A. Classical Creutzfeldt-Jakob Disease

Although the mode of action of prion diseases has been elucidated largely due to public urging because of the enormous publicity surrounding the outbreaks of BSE and vCJD, they are by no means the only prion diseases known. In fact, vCJD is still extremely rare when compared to classical CJD, which strikes one in a million people.

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[94] Liebman, S., **2002**. Progress toward an Ultimate Proof of the Prion Hypothesis, **The Proceedings of the National Academy of Sciences of the USA**, 99, pp. 9098-9100.
[95] Maddelein, M., et al., **2002**. Amyloid aggregates of the HET-s prion protein are infectious, **The Proceedings of the National Academy of Sciences of the USA**, 99, pp. 7402-7407.

There are three known types of CJD (genetic, iatrogenic and sporadic) distinguished by the mode of acquisition of the disease.

Genetic CJD occurs in families that carry mutations in the Prp gene, theorized to encode PrP proteins that have a high rate of spontaneous misfolding into a PrP-sc-like form.[96] There are 17 mutations known to predispose patients to genetic CJD, including point mutations (that change one amino acid for another in the encoded protein) and insertions (which add several amino acids into the encoded protein).[97] The symptoms are similar to other types of classical CJD, yet the mean age of onset varies with the particular mutation inherited (varying from 39 to 62 years).[98]

Iatrogenic CJD is caused by accidental transmission of the CJD agent during a medical procedure. Such transmission can be caused by the administration of hormones extracted from the pituitary gland of a cadaver infected with CJD or from cornea or neural grafts from similar sources.[99] Several hundred iatrogenic CJD cases have been reported, prompting the medical community to screen tissue donors for CJD.

CJD that is associated with neither a medical procedure nor family history is called sporadic CJD (but is symptomatically distinct from vCJD). The mean age of onset of sporadic CJD is in the late 60s, with cases younger than 50 being extremely rare.[100] This disease is suspected to arise from spontaneous misfolding of the prion protein into a PrP-sc-like form.

Regardless of the method of acquiring classical CJD, the symptoms are similar. The patient usually presents with neurological symptoms, such as cognitive impairment and ataxia. The disease rapidly progresses and blindness, dementia and uncontrollable limb movements appear.[101] In 6 months most patients are moribund in an immobile and mute final state. The rapid (4-6 month) progression to an immobile final state and initial symptoms lacking psychiatric disturbance distinguishes classical CJD from vCJD.

Like vCJD, the brains of patients with classical CJD show accumulation of PrP-sc upon autopsy. Before death, CJD patients often present with unusual neural activity by

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[96] Knight, R and Collins, S., **2001.**
[97] Ibid.
[98] Ibid.
[99] Brown, P. et al., **1992.** "Friendly Fire" in Medicine: Hormones, Homografts, and Creutzfeldt-Jakob Disease, **The Lancet**, 340, pp. 24-27.
[100] Knight, R. and Collins, S., **2001.**
[101] Ibid.

electroencephalogram and 14-3-3 protein (a marker for neurological damage) is often present in the cerebrospinal fluid.[102,103] Neither of these signs appear frequently in vCJD patients.

4B. Gerstmann-Straussler-Scheinker Syndrome (GSS)

GSS is contracted only through the inheritance of mutated Prp alleles; all documented cases have relatives afflicted with GSS. There are several mutations in the Prp gene that can lead to GSS, seven point mutations and 3 insertions.[104] GSS is only weakly transmissible by the injection of infected material into other animals, unlike CJD or vCJD.[105]

The mean age of onset of GSS is 50 years but some patients are as young as 25. The illness lasts for, on average, five years, distinguishing it from both vCJD and CJD.[106] GSS is characterized by a generalized and gradual neurological deterioration, both in motor skills and cognition. Similar to vCJD, patients suffering from GSS will suffer from uncontrollable rage, irritability and depression. Similar to CJD, moribund patients are immobile and mute.

Like all other human prion diseases, accumulation of PrP-sc is observed in the brain upon autopsy. Like vCJD, magnetic imaging of the brain is of little diagnostic use.[107]

4C. Fatal Familial Insomnia (FFI)

FFI is the rarest and most recently described prion-linked neurological disorder. It is only transmitted by inheritance of a mutant Prp allele. Five point mutations, one nonsense mutation (which causes a truncated protein to be made) and one insertion

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[102] Steinhoff, B. et al., **1996.** Accuracy and Reliability of Periodic Sharp Wave Complexes in Creutzfeldt-Jakob Disease, **The Archives of Neurology**, 53, pp. 162-165.
[103] Zerr, I. et al., **1998.** Detection of 14-3-3 Protein in the Cerebrospinal Fluid Supports the Diagnosis of Creutzfeldt-Jakob Disease, **The Annals of Neurology**, 43, pp. 32-40.
[104] Knight, R. and Collins, S., **2001.**
[105] Tateishi, J. et al., **1996.** Experimental Transmission of Creutzfeldt-Jakob Disease and Related Diseases to Rodents, **Neurology**, 46, pp. 532-537.
[106] Knight, R. and Collins, S., **2001.**
[107] Brown, P. et al., **1991.** Clinical and Molecular Genetic Study of a Large German Kindred with Gerstmann-Straussler-Scheinker Syndrome, **Neurology**, 41, pp. 375-379.

mutation in the Prp gene are associated with the disease.[108] However, these mutations are incompletely penetrant as several people in an afflicted family have been shown to inherit the mutant alleles yet remain disease free.[109]

FFI usually manifests itself when the patient is in his/her 40s, but can strike patients as young as 20.[110] The patient usually succumbs of the disease in a year after the first symptoms appear. As the name suggests, the primary symptom in FFI is a profound disruption of sleep (due to disruption of circadian endocrine function), with attendant loss of attention and cognition. A variety of physical symptoms appear in the course of the disease, including symptoms associated with the common cold (chills, runny nose, etc.). Ataxia appears relatively late in the course of the disease.[111]

Besides its primary symptom, FFI is most easily distinguished from other prion diseases by a variety of hormonal irregularities. Hormones that are normally controlled by the sleep-wake cycle (such as melatonin) remain abundant regardless of time of day.[112] Close examination of brain-function and REM movements during sleep will also disclose profound disturbance.

4D. Kuru

Kuru is a transmittable prion disease caused by the ingestion of infected material from others who died of kuru. It was largely confined to the Fore-speaking peoples of Papua New Guinea, and accounted for up to 50% of the deaths in certain tribes. Since the outlawing of ritualistic cannibalism in the 1950s, the disease has shown a steady decline.[113] While mourning the death of a relative, it was common practice to eat parts of the body of the dead. In these rituals, men (who only rarely acquired kuru) ate the muscle tissue of the dead, women and children (who were the most frequent to succumb to kuru)

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[108] Reder, A. et al., **1995.** Clinical and Genetic Studies of Fatal Familial Insomnia, **Neurology**, 45, pp. 1326-1328.
[109] Medori, R. et al., **1992.** Fatal Familial Insomnia, a Prion Disease with a Mutation at Codon 178 of the Prion Protein Gene, **The New England Journal of Medicine**, 326, pp. 444-449.
[110] Medori, R. et al., **1992.**
[111] Ibid.
[112] Reder, A. et al., **1995.**
[113] Gajdusek, D., **1977**, Unconventional Viruses and the Origin and Disappearance of Kuru, **Science**, 197, pp. 943-960.

tended to eat the less desirable, and more infectious, tissues such as the brain.[114] There is no evidence of kuru being transmitted from afflicted mother to newborn or fetus.

Kuru manifests with unsteadiness while walking, with gradual decrease in coordination as the disease progresses. When the patient is capable of movement, it is characterized by tremors and shivering. Within 9 months, the disease progresses to inhibit all movement and the patient will usually die of starvation or other secondary causes due to the inability to move (static bronchopneumonia or infected pressure sores).[115] There seems to be little cognitive impairment in victims of kuru. In contrast to other prion diseases, usually marked by depression in the afflicted, patients with kuru often seem euphoric.

4E. Prion Diseases Afflicting Other Organisms

A variety of prion diseases are now known. Several species have been diagnosed with newly emergent prion diseases after being fed meat infected with the BSE agent. However, some other prion diseases are caused by factors other than exposure to BSE. The most famous of which is chronic wasting disease, a transmissible prion disease that afflicts wild ruminants such as deer and elk. Like scrapie, chronic wasting disease is horizontally transmitted through an unknown mechanism, although consumption of the chorioallantoic membrane ejected from an afflicted mother after birth seems likely. Thus far, chronic wasting disease is largely confined to North America. Although no evidence exists that chronic wasting disease can transmit to mankind, the prevalence of the disease has hurt the industry that raise these animals in captivity.[116]

Mink are afflicted by transmissible mink encephalopathy. Although symptoms of this disease are similar to BSE and other prion diseases, the mechanism of transmission remains completely obscure.

Although not strictly 'pathogens", prions affect other organisms outside the animal kingdoms. Two phenotypes in yeast are transmitted by prions via the sharing of cytoplasm (either through mating or replication). The URE3 prion disrupts normal metabolic regulation by nitrogen and the PSI prion allows moderate nonsense mutation

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[114] Gajdusek, D., **1977.**
[115] Ibid.
[116] APHIS, **2001.** Chronic Wasting Disease, **APHIS Factsheets.**

suppression. Both prions act in a manner similar to that hypothesized for the PrP protein. The prions are folded in a protease resistant form that transmute its cellular homolog into a similar form. These prion-like proteins then aggregate and lose their function, allowing the prions to soak up all the normally functional protein from the cell.[117] Due to their genetic tractability, yeast prions have enabled basic research into the nature of prion diseases and helped define their mode of action. A prion in the fungus, *P. anserina*, cuases mating incompatibility and experiments with this prion strongly support the prion hypothesis (see Case for Associated Virus, above).

Table 4. Characteristics of prion diseases other than BSE or vCJD

Other Prion Disease	Characterisitics
All TSEs	Progressive and fatal neurological decay Transmissible if brain-material is transferred to another host Accumulation of PrP-sc prior to death
Classical CJD	Rapid decline after first symptoms recognized Can be inherited, sporadic or iatrogenic Except for iatrogenic cases, only strikes the elderly Worldwide-distribution
GSS	Extremely rare Only inherited Can afflict patients in their 30s
Familial Fatal Insomnia	Extremely rare Only inherited Primarily afflicts patients in their 40s Characterized by pronounced sleep distrubance
Kuru	Confined to Fore-speaking tribes of New Guinea Caused by eating the brains of the dead Primarily struck women and children
Chronic Wasting Disease	Affects deer and elk in North America Spreading Rapidly Method of transmission is obscure
Transmissible Mink Encephalopathy	Method of transmission is obscure Affects captive mink
Fungal prions	URE3—disrupts metabolism PSI—causes nonsense suppression *P. anserina* prion can be made sythetically

5. Difficulties with the Detection and Treatment of Prion Diseases

Diagnostic medicine today relies on three general assays to determine if a patient is harboring an infectious microbe. Bacteria, fungi and parasites can be cultured from samples taken from the patient. Microorganisms and viruses can be detected through the repeated amplification of their genomes by the polymerase chain reaction, a technique

[117] Taylor, K. and Wickner, R. Prions, a Challenge for Science, Medicine and Public Health System, **Contributions to Microbiology**, 7, pp. 21-31.

sensitive enough to detect the presence of one viral genome in a sample. The polymerase chain reaction is also used to detect microbes in inert samples. Any infection by a pathogen also produces an immunological response in the host, identification of antibodies in the serum of patients can lead to rapid diagnosis of infection.

In contrast, diagnostics based on any of the classical techniques are of little use in the diagnosis of prion diseases. The absence of nucleic acid associated with prion diseases prevents the use of the polymerase chain reaction to identify prion contamination. The lack of an immunological reaction by the host prevents antibody-based diagnostic techniques in the diagnosis of prion diseases. Worse still, the dose PrP-sc required to cause lethal disease in half of mice exposed is roughly only 10^{-15} grams per kilogram of body weight (compared to 10^{-10} grams per kilogram of body weight for botulin toxin A—the next most lethal protein known).[118] This extreme infectivity requires extremely sensitive assays to detect the presence of potentially lethal contaminations.

The lack of diagnostics to identify pre-symptomatic, but exposed patients creates two difficulties. By the time symptoms appear in humans, significant neuropathology has developed. Treating these patients will not only require the reversal of prion accumulation but a regeneration of neural tissue. The lack of diagnostics limits the use of therapeutics that would prevent the buildup of PrP-sc, neuronal death in response to PrP-sc buildup or the delivery of PrP-sc to the brain. The lack of diagnostics also increases the uncertainty in the safety of products derived from organisms potentially infected with prions. At the present time, methods to detect contamination by PrP-sc in food are very rudimentary. Possible, though unlikely, contamination of PrP-sc in human blood has lead to a constriction of the supply in the US.[119] Rudimentary diagnostics also leaves the question of the extent of the BSE epidemic uncertain; possibly hundreds of thousands of people are currently incubating vCJD, increasing the probability of iatrogenic infections.

Table 5. Difficulties associated with diagnosis and detection of prion diseases

Difficulties associated with diagnosis and detection of prion diseases
Lack of nucleic acid prevents conventional magnification procedures
Lack of immune response in host prevents classical serological procedures
Extreme toxicity requires extremely sensitive detection of agent to determine contamination

[118] Bolton, D. et al., **1987.** Isolation and Structural Studies of the Intact Scrapie Agent Protein, **The Archives of Biochemistry and Biophysics,** 258, pp. 579-590.
[119] Casagrande, R., **2001.**

6. Summary

Table 6. Summary of introduction

Characterisitics of BSE	Progressive, fatal neurological disease of cattle Acquired by the consumption of infected tissue in feed Presence is devastating to beef and diary markets Probably originated from scrapie
Characteristics of vCJD	Progressive, fatal neurological disease of mankind There is no current treatment of cure One of several prion diseases of mankind Probably originated from the consumption of infected material derived from cattle
Evidence that BSE and vCJD agent are the same	PrP-sc isolated from BSE infected cattle and vCJD infected people share similar biochemical properties vCJD first appeared after the BSE epidemic Other animals get TSEs from BSE infected cattle Humanized mice can contract BSE Symptoms of mice infected with BSE and vCJD are the identical
Evidence against viral involvement in BSE and vCJD	Infectivity is resistant to treatments that destroy nucleic acid, an essential component of viruses No immunological response mounted by host No viruses are observed in infectious material No nucleic acid of sufficient length to support a viral genome is found in infectious material
Evidence that prions cause BSE and vCJD	PrP-sc is (almost always) associated with the diseases Animals lacking the Prp gene are resistant to infection Animals overexpressing PrP-c are hyper-sensitive to infection Unfolding of PrP-sc in infectious material abolishes infectivity Transmutation of PrP-c into a PrP-sc like state is observable in vitro
Characteristics of PrP-c	Protease sensitive, plasma membrane protein Secondary structure is dominated by α-helices Binds copper and has superoxide-dismutase activity
Characteristics of PrP-sc	Resistant to enzymes that destroy proteases Tends to aggregate Secondary structure is dominated by β-sheet Converts PrP-c into an a PrP-sc-like form

TECHNOLOGY ASSESSMENT AND SUGGESTIONS FOR FUTURE DEVELOPMENT

1. Detection of Prion Contamination in Samples

Even though there is some doubt that prions are actually the cause of BSE and vCJD (see The Case for an Associated Virus) there are several reasons why it is essential to detect prions where they occur. Primarily, prions are the most likely cause of BSE and vCJD as no other pathogens have been found that have the characteristics required to transmit these diseases. Secondly, prions are found (in all but a few cases) associated with the disease and the infectious material. Thirdly, treatments that destroy prions destroy infectivity of a sample. For all these reasons, it remains the best option to focus on the detection of prions as there are no other markers that correlate as highly with infectivity.

1A. State of the Art

Test for CNS contamination—RIDASCREEN, R-Biopharm Inc.[120] and an unnamed test from ABC Research Corporation[121].

This test does not look for PrP-sc itself, but instead is used to detect central nervous system tissue that may contaminate foodstuffs. Since central nervous system tissues are the primary source of BSE-infective material, this test can be used to identify products which *may be* a risk-factor in the transmission of BSE. Elimianting feed with contamination from central nervous system tissues will greatly reduce the chance of transmission of BSE. The basis of both tests is an enzyme-linked immunosorbent assay (ELISA) that uses antibodies specific for glial fibrillary acidic protein, a protein present only in the tissues of the central nervous system.[122] These ELISA-based tests require only 15 minutes to complete and can detect as little as 0.1% central nervous system tissue contamination.[123] Other markers have been suggested in addition to glial fibrillary acidic

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[120] See R-Biopharm News: http://www.r-biopharm.com/Rida/News_pdf/News_I_01_engl.pdf. pages 1-2.
[121] See ABC's press release: http://www.abcr.com/PressRelease01.asp
[122] Schmidt, G., **1999.** An Enzyme-linked Immunosorbent Assay for Glial Fibrillary Acidic Protein as an Indicator for the Presence of Brain or Spinal Cord in Meat, **The Journal of Food Protection**, 62, pp.390-393.
[123] Schmidt, G., **1999.**

protein, including neuron specific enolase.[124] Others have suggested that, since central nervous tissue has extremely high levels of cholesterol and fatty acids, material with greater than a threshold amount of either compound be rejected as risky.[125,126] The benefit of a test that measures cholesterol levels is that it is extremely rapid (less than 5 minutes) the drawback, of course is that several feeds (such as those made from recycled fryer grease) would falsely indicate central nervous tissue contamination. In a laboratory mock-up of the meat packing industry, tests that measured central nervous tissue contamination in food performed well, detecting tissue contamination as low at 0.25% repeatedly.[127]

Because of their speed and simplicity, these tests are valuable for screening out feed that is potentially contaminated with material that naturally contains the highest amount of BSE material. Interestingly, this test could detect potentially infectious material whether or not PrP-sc is proven to be the causative agent of BSE. However, these tests are insensitive, infectivity can be carried in less than a microgram of brain (these tests can detect only about a milligram of central nervous tissue). These tests cannot detect the contamination of feed with tissues from the lymphoreticular system (which have been shown to harbor significant infectivity) or purified PrP-sc.

Biochemical Detection of PrP-sc

PrP-sc may not be present in samples that have central nervous tissue and PrP-sc could be in samples where central nervous tissue is not present. To overcome this shortcoming of the tests described above, several diagnostic kits on the market today directly test for the presence of PrP-sc (produced by E.G.& G. Wallac Ltd, Prionics AG, Enfer Ltd., and BioRad). Despite a few claims to the contrary, there are currently no antibodies that specifically recognize PrP-sc in the presence of PrP-sen. To detect the

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[124] Lucker, E., **1999.** Development of an Integrated Proceedure for the Detection of Central Nervous Tissue in Meat Products Using Cholesterol and NSE and Markers, **The Journal of Food Protection,** 62, pp. 268-276.

[125] Lucker, E., **1999.**

[126] Niederer, M and Bollhalder, R., **2001.** Identification of Species Specific Central Nervous Tissue by Gas Chromatography- Mass Spectrometry (GC-MS)—a Possible Method for Supervision of Meat Products and Cosmetics, **Mitteilungen Lebensmittel Hygiene,** 92, pp. 133-144.

[127] Lucker, E. et al., **2002.** Detection of CNS and PrPsc in Meat Products, **Berliner und Munchiner Tierarztliche Wirtschrift,** 115, pp. 111-117.

presence of PrP-sc in the presence of PrP-sen, these kits take advantage of the differential protease sensitivity of the two forms. Samples are treated with proteases and the remaining protein is used in either a western blot or ELISA to identify any PrP protein remaining. In tests analyzing tissue taken from the brains of scrapie infected or control sheep, the most sensitive test was able to detect the presence of PrP-sc in .0025 g of brain.[128] The majority of these tests are quite specific, only detecting PrP-sc in samples actually infected with PrP-sc.[129] The most rapid of these tests require 4 hours to complete.[130]

These tests can be used to detect PrP-sc in food, carcasses or medical samples. They are relatively rapid and sensitive but need to be performed by a trained laboratory technician, which increases the test's effective cost and limits their widespread use. Furthermore, these test cannot distinguish between PrP-res (which is protease resistant, can be made synthetically, but is non-pathogenic) and PrP-sc, and could lead to false alarms.

Animal Bioassays

The gold standard of assays to detect infectivity is the animal bioassay, a direct and extremely sensitive test for infectivity of a sample. The sample is homogenized and then injected directly into the experimental animal's brain. Experimental animals used are either Syrian golden hamsters, mice or cows. Rodents have the advantage of low cost and maintenance; cows however, are at least two orders of magnitude more sensitive to inoculation with ether BSE or vCJD homogenate. Experimental animals used today are mice that lack the murine Prp gene and have the bovine Prp gene instead, combining low maintenance costs with increased sensitivity.

The pathogenicity of the sample is assayed in two ways: monitoring the symptoms of infected animals or performing biochemical analysis on brain samples from animals after injection. Symptomatic monitoring requires that the clinician allow the disease to run its course until symptoms develop (which takes approximately 270 days).

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[128] European Commission, Directorate B—Scientific Health Opinions, **1999.** The Evaluation of Tests for the Diagnosis of Transmissible Spongiform Encephalopthy in Bovines.
[129] European Commission, **1999.**
[130] Ibid.

In contrast, PrP-sc will be detectable by western or ELISA (as above) 30 days after intercerebral inoculation. Monitoring of experimental animals for symptoms to appear requires little technical expertise (except for the inter-cerebral injection) but the long incubation time of the disease severely limits utility. The strength of these assays is that they directly measure pathogenicity of the sample, there is little possibility for false positives. Inoculation followed by western blotting increases the sensitivity of the biochemical assay still requires technical expertise and a prohibitively long experimental time. Furthermore, as noted above, some mice will become infected with a BSE-like disease without the accumulation of PrP-sc in the brain, giving the biochemical-bioassay test a small, false negative rate.[131] Due to the fact that PrP-sc injected intercerebrally is several orders of magnitude more pathogenic than PrP-sc ingested, these assays can detect contamination at lower levels than would cause infection in animals in the field.

Fluorescent Correlative Spectroscopy

This method measures the relative size of fluorescent particles in a sample and thereby takes advantage of the fact that PrP-sc causes PrP-sen to change form and aggregate.[132] A sample is interrogated by a tight laser beam. As fluorescent particles enter the beam they emit fluorescence. The time it takes each particle to travel the width of the beam is measured and directly correlates with its size (Brownian motion and diffusion rate are both inversely related to size). Therefore, the bigger the particle, the slower it moves and the longer it stays in the beam.

Each sample is added to a solution that contains fluorescently labeled PrP-sen. If the sample contains PrP-sc (or PrP-res) fluorescent PrP-sen will transmute and aggregate. This aggregation will cause each fluorescently labeled particle to increase in size and therefore take more time to travel across the laser beam, a difference detectable by the spectrometer. In theory, this device can be used to detect concentrations of aggregated proteins at approximately 10^{-15} molar (or 10^{-14} grams per milliliter for PrP protein). Difficulties inherent with working with PrP protein (including self-aggregation of PrP-

[131] Lasmezas, C. et al., **1997.** Transmission of the BSE Agent to Mice in the Absence of Detectable Abnormal Prion Protein, **Science**, 274, pp. 402-404.
[132] Bieschke, J. et al., **2000.** Ultrasensitive Detection of Pathological Prion Protein Aggregates by Dual-color Scanning for Intensely Fluorescent Targets, **The Proceedings of the National Academy of Sciences of the USA,** 97, pp. 5468-5473.

sen) limited the detection to roughly 10^{-8} grams per milliliter.[133] Furthermore, other technical problems give a high false positive rate (approximately 30%) limiting the usefulness of this technique. This technique is relatively new and many groups are working to improve its reliability and sensitivity.

Even if it were working at theoretical performance limits, there are a few limitations of this device. Primarily, the fluorescence correlative spectroscope is an extremely complicated machine. The expense of each device prohibits its use in many diagnostic labs. Furthermore, the machine has to be run and maintained by a highly trained operator. Since this device keys on aggregation as a readout of infectivity, PrP-res may be confused with PrP-sc, leading to false positives. However, improved versions of this device would be perfectly suited to be used in national diagnostic laboratories where suspect samples could be sent for rapid diagnosis.

Table 7. State of the art in prion detection

Method	Advantages	Disadvantages
Detection of central nervous tissue contamination	Rapid (15 min) Relatively cheap	Nervous tissue contamination does not imply PrP-sc contamination Requires biochemical expertise Relatively insensitive (detects roughly 0.1% contamination)
Biochemical detection of PrP-sc	Low false positive rate Specific for PrP-sc or PrP-res Relatively cheap	Requires biochemical expertise Cannot distinguish between PrP-sc or PrP-res Time consuming (4 hours at least) Not sensitive enough to detect dangerous amounts of PrP-sc
Animal bioassays	Extremely sensitive Specific for pathogenic sample	Extremely time consuming (30 to 270 days) In biochemical bioassay, some rate of false negatives Expensive due to costs of animal maintenance
Fluorescence correlative spectroscopy	Sensitive	**Requires rarified expertise** **Device is expensive** **High rate of false positives** **Cannot distinguish between PrP-sc and PrP-res**

1B. Options to Improve Existing Assays

Prions are pathogenic even when dilute. Possibly, animals may accumulate a lethal dose of the BSE agent over time if their food contains low levels of PrP-sc. The combination of the extreme toxicity of PrP-sc with the possibility that sub-lethal doses may accumulate to a lethal level in an animal over time requires that tests be extremely

[133] Bieschke, J. et al., **2000.**

sensitive to detect even minute amounts of the BSE agent in products given to animals or people.

Pre-concentration of PrP-sc by Precipitation

One way to detect dilute PrP-sc is to concentrate the prions from a large volume through precipitation. Researchers have found that several compounds are able to precipitate PrP-sc out of solutions, enabling the concentration of the prion from samples that may be otherwise too dilute to analyze rigorously. At pH 5, almost all PrP protein in a sample precipitates. [134] Equally effective at precipitating PrP protein is a solution containing 25% ethanol with 0.25 M sodium chloride.[135] Others have found that solutions containing 0.3% phosphotungstic acid precipitates PrP protein equally well but requires more expensive reagents and some manipulation of the sample prior to acid treatment.[136]

Precipitation prior to analysis is obviously advantageous to concentrate prions into amounts that exceed the limits of detection. Also, precipitation allows the analysis of large samples (for example a 100kg portion of feed from a shipment) in highly sensitive and small-scale assays. The scale at which the food industry provides material requires rapid and simple means to analyze extremely large volumes of products.

Precipitation (using either the acidic or salt/ethanol solution) should be done prior to the analysis of any sample too large to be immediately handled by a single assay. After precipitation, commercial tests could be used, gaining in sensitivity.[137] For maximum food security, a portion of the pooled blood from abattoirs can be treated to allow precipitation and analysis of PrP-sc. The sheer volume of blood produced limits the use of current detection methodologies without a previous concentration step. If provided as a pre-made and concentrated solution, unskilled operators could add a predetermined amount of precipitant to a sample prior to more demanding biochemical analysis, thereby putting no further demand on skilled technicians.

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[134] Cai, K. et al., **2002.** Solvent-dependent Precipitation of Prion Protein, **Biochemica et Physica Acta,** 1597, pp. 28-35.
[135] Cai, K. et al., **2002.**
[136] Wadsworth, J. et al., **2001.** Tissue Distribution of Protease Resistant Prion Protein in Variant Creutzfeldt-Jakob Disease Using a Highly Sensitive Immunoblotting Assay, **The Lancet,** 358, pp.171-180.
[137] Wadsworth, J. et al., **2001.**

Cyclic Magnification of PrP-sc

The polymerase chain reaction has greatly advanced medical diagnostic capability by enabling the magnification of extremely dilute quantities of nucleic acid. In theory, this method allows the magnification and subsequent identification of even one pathogen in a sample. Unfortunately, the polymerase chain reaction cannot work on prions because no nucleic acid has been consistently found associated with infectivity. However, researchers have devised a cyclic amplification process for PrP-sc that may enable magnification of prion contaminated samples.[138]

A sample potentially contaminated with PrP-sc is added to a solution containing abundant PrP-sen (initial experiments used brain homogenate from a healthy animal). If there is PrP-sc in the sample, it will convert some of the PrP-sen into PrP-sc. Sonication is then used to release and distribute the newly magnified PrP-sc throughout the solution and a second cycle of transmutation is begun, followed by sonication. Each cycle takes 1 hour and approximately 10 to 20 cycles provides optimum magnification. This assay was used to amplify undetectable amounts of PrP-sc to amounts detectable by conventional methods.[139]

When sensitivity is desired over rapidity, this method should be used to magnify otherwise undetectable amounts of PrP-sc. After magnification, currently used tests could be performed with greater sensitivity. The semi-automated nature of this method enables those with limited technical expertise to perform it, putting little further demand on skilled technicians.

Microfluidic-based assays

A microfluidic-based assay is one that involves the analysis of sub-microliter volumes of analyte. There are several advantages inherent in microfluidics versus conventional assays. Primarily, the small volumes analyzed increase the absolute sensitivity of the assay. For example, if the level of detection of a particular assay is 1 M of a 1000 dalton analyte, there would need to be 1000g of the analyte in one liter for detection to be possible. If the volume of sample was 1 microliter, there would only have

[138] Saborio, G., et al., **2001.** Sensitive Detection of Pathological Prion Protein by Cyclic Amplification of Protein Misfolding, **Nature,** 411, pp.810-813.
[139] Saborio, G. et al., **2001.**

to be 1 mg of analyte for detection. Other advantages of microfluidics include: a smaller sample facilitates less-intrusive medical testing, small device size increases portability while decreasing reagent use and power drain (for automated devices), and microfludics are readily interfaced with commercially available pumps for easy automation while maintaining portability.

The current assay formats can be readily adapted to microfluidic formats. ELISA, for example, can be removed from the tissue culture dish and placed into an automated microfluidic format. The immobilized antibody is deposited in a microfluidic channel. The operator then places the sample (protease treated to remove PrP-sen) in a reservoir and it is drawn through the channel with the immobilized antibodies. As the analyte passes over the antibodies, it is bound and unbound material washes past with the rest of the sample. Wash buffer and secondary reagents can then be pumped over the same region in an automated fashion for detection. The use of microfluidics reduces the operator burden by increasing the automation feasibility. Note that pre-concentration by precipitation may be required for the sample volume to be compatible with the small volumes of microfluidics.

Molecules that can Bind PrP-sc Preferentially to PrP-sen

Despite some early reports, there are no antibodies that bind to PrP-sc and not PrP-sen. The existence of these reagents would greatly facilitate most assays for the detection of PrP-sc because many samples contain overwhelming amounts of PrP-sen and no PrP-sc. Therefore, PrP-sen must be eliminated prior to analysis or false positives would result. The most common way of destroying the PrP-sen prior to analysis is via treatment with proteases, a time consuming step and one that often does not go to completion, leading to false positives due to survival of PrP-sen. If a reagent existed that bound to PrP-sc and not PrP-sen, the protease treatment step could be skipped; assay speed would increase and the rate of false positives would decrease. Because antibodies (and related molecules) can distinguish between two folding states of the same protein, the generation of reagents specific for PrP-sc and not PrP-sen is definitely possible.

Researchers have found that plasminogen, a pro-protease found in plasma, binds to PrP-sc but not PrP-sen.[140,141] Plasminogen could be used instead of the capture antibody in an ELISA or instead of the primary antibody in a western blot (and where the secondary antibody binds to plasminogen). However, the strength of binding of plasminogen to PrP-sc has not been characterized and the interaction may not be of sufficient strength for use in ELISAs or western blot assays.

Attempts to generate an antibody specific for PrP-sc though conventional antibody-generation protocols have failed. The value of this reagent (in time and specificity gained) justifies more experiments to generate these antibodies. Two protocols could be pursued: phage-display for Fab (the target-binding domain of antibodies) generation, or selective processing for aptamer generation.

In phage display, the genome of bacteriophage viruses is modified so that the virus displays various Fabs on its surface. Through a process of multiple rounds of selection, Fabs can be developed that strongly bind to the reagent of interest; these Fabs can then be incorporated into full antibodies through conventional molecular biological methods. In this instance, the phage are exposed to immobilized PrP-sen. All phage that display Fabs that enable them to bind to PrP-sen are discarded by collecting only the unbound fraction. The remaining phage are then exposed to immobilized PrP-sc. The unbound phage are discarded and the phage that bind to PrP-sc are eluted and magnified by co-culture with bacteria. The bacteria allow the viruses, and thier Fab cargo, to replicate and adjust specificity of the Fab through mutation. Further rounds of negative selection by binding to PrP-sen and positive selection by binding to PrP-sc are then performed. All subsequent rounds of binding to PrP-sc are done at increasing stringency to select Fabs that bind to PrP-sc more strongly. Several rounds of selection and counter-selection should allow the discovery of Fabs that bind selectively to Prp-sc.

The selection of aptamers, molecules of RNA that bind to targets with specificity and strength similar to antibodies, can proceed in a similar manner. Libraries of millions of sequences of RNA are made by conventional methods in a nucleic acid synthesizer.

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[140] Fischer, M. et al., **2000.** Binding of Disease-assoicated Prion Protein to Plasminogen, **Nature,** 408, pp. 479-483.

[141] Maissen, M. et al., **2001.** Plasminogen Binds to Disease-assoicated Prion Protein of Multiple Species, **The Lancet**, 357, pp. 2026-2028.

The majority of these molecules displays enormous diversity while the ends of the molecules are identical. The library of aptamers is exposed to PrP-sen and all aptamers that bind are discarded. Following this negative selection, apatmers are selected for binding to PrP-sc (in a manner analogous to the phage display technique described above). After positive selection, the remaining aptamers are magnified through mutagenic polymerase chain reaction using primers that anneal to the conserved ends of the aptamers. In this way, only aptamers that bind to PrP-sc but not PrP-sen survive to be magnified and altered slightly for the next (and more stringent) round of selection. In several rounds of this selection, reagents that specifically bind to PrP-sc but not PrP-sen could be generated.

All four of the advances suggested above could be used in combination to greatly improve current assays. First, a large sample is concentrated by precipitation. This precipitated sample is then amplified (if the time requirements can be justified) through the cyclic protocol of aggregation and sonication. The concentrated, amplified sample is then analyzed in an ELISA in a microfluidic format, increasing sensitivity and decreasing the burden on skilled technicians. The inclusion of reagents specific for PrP-sc instead of PrP-sen would eliminate the need for protease pre-treament, increasing the sensitivity of the assay while decreasing assay time. Note, however, that these improvements still rely on (and have some of the limitations of) conventional assays to detect PrP-sc. In the next section, assays for the detection of TSE infectivity will be described based on other methods.

Table 8. Suggestions to improve existing methods of prion detection

Improvement	Advantage
Precipitation	Increases sensitivity of base assay Enables analysis of large samples Simple and rapid
Cyclic Amplification of PrP-sc	Increases sensitivity at the expense of time
Microfluidic-based Analysis	Increases sensitivity of base assay Decreases burden on operator due to facility of automation Requires smaller sample volumes Decreases reagent requirements
Reagents Specific for PrP-sc	Decreases time requirements of biochemical assays by eliminating need for protease treatment Decreases false positive rate of assay for same reason

1C. Other Potential Methods for the Detection of PrP-sc

Analysis of the Metal Content of PrP Proteins

By moving away from the conventional assays, the protease treatment step (with its undesirable characteristics) can be avoided. As mentioned in the introduction, PrP-sen binds to copper whereas PrP-sc binds to manganese and zinc.[142] The PrP-sc content of a sample can be analyzed by first capturing all PrP proteins (both PrP-sc and PrP-sen) and then eluting the captured proteins into a device capable of identifying metals in a sample (Raman spectrometer, electron paramagnetic resonance detector, flame spectrophotometer, or inductively-cooled mass spectrometer). The presence of manganese or zinc in the sample would indicate the presence of PrP-sc. Some of these devices (like the flame spectrophotometer) are highly-automated and relatively easy to use. This method eliminates both the protease treatment and arduous and reagent intensive detection steps of ELISAs and western blots (as elemental analysis can be quite rapid and requires no additional reagents for detection). By preceding the metal analysis with precipitation and cyclic magnification of PrP-sc, this assay could be as sensitive and much more rapid than conventional assays.

Detection of PrP-sc Through Plasminogen Activation

As mentioned above, PrP-sc (and not PrP-sen) binds to plasminogen.[143] This binding leads to the activation of plasminogen through another enzyme (uPA or tPA).[144] The activation of plasminogen leads to the cleavage of plasminogen and yields plasmin, an active protease. To detect the presence of PrP-sc, a sample containing plasminogen and its activator, tPA, can be incubated with the analyte. The production of plasmin can be measured through the cleavage of a fluorogenic plasmin substrate, H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (which is fluorescent only after cleavage by plasmin).[145] Fluorescence produced after the sample and analyte are mixed indicates the cleavage of the substrate of plasmin and therefore the activation of plasminogen, hence the presence

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[142] Lehmann, S., **2002**. Metal Ions and Prion Disease, **Current Opinion in Chemical Biology**, 6, pp. 187-192.
[143] Fischer, M. et al., **2000.**
[144] Ellis, V. et al., **2002.** Plasminogen Activation is Stimulated by Prion Protein and Regulated in a Copper-Dependent Manner, **Biochemistry**, 41, pp/ 6891-6896.
[145] Ellis, V. et al., **2002.**

of PrP-sc. This activation of plasminogen via PrP-sc is extremely rapid, enabling detection in several minutes. However, it is unclear whether components of other samples (blood, brain material) will also activate plasminogen to a certain extent, thereby increasing the background and reducing the sensitivity to PrP-sc. If high background is a factor, the sample can be pre-treated with proteases, as in conventional assays, to destroy other activating proteins.

Even if protease pre-treatment is required to reduce the background generated by biological samples, this fluorescent analysis is still more rapid and easier to perform than other biochemical assays. Furthermore, since PrP-sc is being detected via an enzymatic activation step, this assay is, in theory, more sensitive than the direct detection of PrP-sc through antibodies (because one PrP-sc molecule could activate several molecules of plasminogen). Furthermore, plasminogen and the substrate of plasmin are more stable than detection reagents used in other assays.

Detection of BSE or vCJD Infectivity via Macrophage Proliferation

The two novel assays described above depend on the detection of PrP-sc. PrP-sc is the only marker associated with BSE infectivity but is not necessarily the causative agent of BSE. Bioassays that measure the body's response to the BSE pathogen are the only way to detect the presence of the pathogen without relying on the detection of PrP-sc (since there is no other known marker of infectivity). One such ex-vivo bioassay takes advantage of the fact that macrophages will proliferate in response to the presence of peptides derived from pathogenic prions.[146] Upon exposure to the analyte, macrophage growth could be measured in several ways.

The cells could be fed radioactive or fluorescent nucleotides, which would become incorporated into their DNA when the cells proliferate. Unincorporated nucleotides would be washed away. The sequestration of the radioactivity or fluorescence inside the cell would indicate that the cells have proliferated and that there are pathogenic prions in the sample. Alternatively, macrophages could be tethered to the assay surface such that cells cover the entire area. As cells proliferate, they can no longer stick to the

[146] Hamilton, J. et al., **2002**. Alzheimer's Disease Amyloid Beta and Prion Protein Amyloidogenic Peptides Promote Macrohpage Survival, DNA synthesis and Enhcanced Proliferative Response to CSF-1 (M-CSF), **Brain Research**, 940, pp. 49-54.

surface (because there is no room) and are released from the surface. After washing, any cells released into the media would indicate that the macrophages have proliferated and that the sample contains potentially infectious material. This type of assay is ideally suited for the microfluidic format.

This test may be rapid, as proliferation could be measured in a matter of minutes, and the test may need unskilled operators, if the assay is sufficiently integrated with microfluidics, but it is non-specific. There are many compounds in a sample that may cause macrophages to proliferate (including macrophage growth factor and certain bacteria). Each sample type would have to be pre-screened for the lack of macrophage stimulating activity before use in this assay. Because of the non-specific nature of this assay, a positive result should be followed by a more specific assay (perhaps a mouse bioassay). Also, any assay that involves living cells as a sensing reagent is expensive and largely unsuitable for storage, limiting the use of this assay in many situations.

Use of Sentinel Cells to Detect BSE or vCJD Infectivity

Animals are more expensive than cells to maintain and the animal bioassays described above take at least a month to complete. With the proper analysis, cells in culture could supplement or replace the bioassay, providing a cheaper, more rapid and equally accurate prediction of BSE infectivity. At present we lack the proper data to develop this assay.

To develop this assay, a genome microarray experiment must be performed. In this type of experiment, pieces of the thousand most highly expressed genes from leukocytes (the first known reservoir of PrP-sc in the body after ingestion) are arrayed on a silicon chip. The leukocytes are exposed for 24 hours to samples containing material derived from a BSE infected cow or a healthy cow. The mRNA of these cells is harvested, made into cDNA and modified with fluorescent dyes (red for the control cow, green for the BSE cow). These labeled cDNA pools are then hybridized to the chip. If cDNA is present from a particular gene represented on the chip, it will hybridize to the spot on the chip corresponding to its complementary sequence. The relative amounts of each mRNA for each gene on the chip is measured through the relative levels of green or red fluorescence where each gene was spotted. Through the genome array experiment,

genes that are up- or down-regulated in response to the presence of BSE infectious material can be identified. Once a set of genes that change expression levels in response to the presence of pathogenic prions is found, an assay based on this information can be developed.

To determine if a sample contains BSE infectious material, leukocytes are exposed to the sample for 24 hours. After this period, the mRNA from the cells is harvested and transcription of the genes known to be regulated by the presence of prions (from the previous experiment) is measured. This measurement can by accomplished by traditional methods (northern blot) or by quantitative and automated reverse-transcriptase polymerase chain reaction. Patterns of gene expression that resemble those from leukocytes exposed to prions would indicate the presence of prions. The sensitivity of this assay stems from the fact that transcriptional changes can be induced by the presence of as few as one molecule. This assay could potentially be used instead of animal bioassays, due to its specificity for BSE infectivity, its lower cost, its more rapid diagnosis and its potential sensitivity.

Alternatively, the readout of this assay could be simplified. The sentinel leukocytes can be stably transfected with reporter genes under the control of the promoters from genes that have their expression altered in a prion dependant manner. Each reporter gene produces a protein that either is an enzyme that can produce a luminescent or fluorescent product or is luminescent, fluorescent itself (for example, acquorin—which is luminescent, green fluorescent protein, or horseradish peroxidase—which can hydrolyse a luminescent substrate). After exposure of the cells to potentially infectious material for 24 hours, the cells are lysated and the lysate is analyzed for the content of reporter proteins by multi-wavelength spectrophotometric analysis. This modification of the transcription assay simply makes the readout easier and more economical.

Transcription can be modulated by many factors. It is possible that other compounds in a sample may alter the expression of some of the same genes affected by BSE infectivity. Prior to the use of this assay on unknowns, the assay must be run on similar control samples to ensure that no artificial modulation of transcription of the target genes will occur.

Table 9. Novel methods to detect prion contamination

Assay	Advantages	Disadvantages
Analysis of Bound Metals	Rapid (5 minutes for binding and washing, 5 mintues for metal analysis) Does not require protease treatment Easily automated No detection reagents	Probably insensitive without prior cyclic amplification Specific for PrP-sc (not BSE infectivity)
Plasminogen Activation	Rapid (5 minutes) Does not require protease treatment Easily automated Probably more sensitive than ELISA or western blots Reagents are cheap and stable	Activation of plasminogen through contaminants in sample may lead to false positives Specific for PrP-sc (not BSE infectivity)
Macrophage Proliferation	Rapid (10 minutes) Sensitive Detects BSE infectivity in addition to PrP-sc	Expensive and requires a lot of maintenance Triggered by any compound that induces macrophage proliferation
Transcription Analysis of Sentinel Cells	Could be extremely sensitive Detects BSE infectivity in addition to PrP-sc More rapid and cheaper than animals bioassays	Expensive Requires experimental work before development Time consuming (requires a day) Requires extensive biochemical expertise Needs to be baselined with new sample types

1D. Reccomendations

Due to their potential utility, reagents (antibodies or aptamers) should be developed that can bind to PrP-sc in preference to PrP-sen. These reagents could be incorporated into current assays, improving the assays in rapidity and specificity.

Due to its potential sensitivity, stability, cost, rapidity and ease of use it is recommended to develop and use the plasminogen activation assay instead of current biochemical assays. This assay could be preceded by PrP-sc precipitation and integrated with microfluidics to increase sensitivity and facilitate automation. Automation potentially enables the use of this assay by the unskilled. This assay could be given to the USDA's rapid response teams (READEO teams) for field analysis of potentially contaminated material or veterinary samples. Food inspectors could be provided with these tests for spot inspection of human food.

Due to its potential sensitivity, speed, cost and rapidity, the transcription analysis assay should be used in addition to conventional animal bioassays. Since the animal bioassay serves as the basis for the definition of infectivity the transcriptional analysis

assay should not replace the animal bioassay but supplement it. On the same day as animal inoculation, the transcriptional analysis assay should be begun to provide specific and sensitive next-day results. Due to the expertise required and high cost relative to simpler assays, the transcriptional analysis assay would be used only in regional diagnostic laboratories for the confirmation of infectivity in highly suspect samples.

For any assay to be useful in the field, the limits of detection and rate of false positives must be determined for every possible material that may be tested and in any condition that the assay may be used. For example, the limit of detection of PrP-sc in a water sample may be lower than the limit of detection in a brain sample due to interference from PrP-sen or other factors in the tissue sample. To validate the assays described above and those suggested in the following sections, it is suggested to perform the following control experiments with each assay:

- Determine the limit of detection and rate of false positives in every material that will be tested (meat samples, blood, brain tissue, urine, grain)
- Each dilution should be performed with PrP-sc, PrP-res, PrP-sen, infected brain tissue and uninfected brain tissue to determine the specificity of the assay in each sample type. False positives should be noted.
- The assays should be performed in various environmental conditions: bright sunlight (which may interfere with fluorescence-based detection) and extremes of heat and cold (which may change the binding properties of reagents.

Diagnosis of BSE in Animals or vCJD in People

2A. State of the Art

Autopsy

The most common method of diagnosing TSEs is upon autopsy. The diagnosis can be made by the discovery of gross or microscopic pathology consistent with the disease (florid plaque associated with gliosis and spongiform appearance of tissue). Alternatively, brain tissue samples can be taken and analyzed for PrP-sc content via a western blot or ELISA as described for sample testing above. Alternatively, brain slices can be fixed with paraformaldahyde, protease treated and in situ immuno-microscopy can

be performed to identify PrP-sc in proximity to brain lesions. All of these tests have the disadvantage of requiring the death of the patient or animal to get the sample. Furthermore, PrP-sc does not accumulate in the brain until relatively late in the course of the disease, which may be too late to prevent spread or provide effective treatment to the patient.

Biopsy/ blood or urine sampling

Several tissues that can be sampled without the death of the subject also accumulate PrP-sc, as detected by conventional methods. The tonsils and the third eyelid all accumulate levels of PrP-sc detectable by the conventional means described above. For the diagnosis of cattle and sheep, PrP-sc has been found also in the leukocytes, but in extremely small amounts only in laboratory tests, requiring sensitive methods of detection. Significant accumulation in the blood appears roughly 2 months post infection in high-dose laboratory trials. In humans and most animal tests, PrP-sc has not yet been identified in the blood of subjects with terminal-stage vCJD or BSE.[147, 148]

In subjects presenting symptoms of vCJD or BSE, PrP-sc accumulates in detectable levels in the tonsils.[149] This test also can distinguish cases of vCJD from CJD as no PrP-sc accumulates in the tonsils of patients affected by CJD. In naturally infected sheep, PrP-sc is first detectable in the tonsils about 10 months after infection (with a mean incubation time of 40 months), indicating that this test could be useful for the pre-symptomatic diagnosis of infected animals.[150]

In studies done on sheep, PrP-sc could be found in the third eyelid (nictitating membrane) prior to the onset of symptoms. Although technically difficult and somewhat painful to the subject, the sampling of this tissue does no harm to the animal. In naturally infected sheep, PrP-sc could be detected in the third eyelid up to 20 months prior to the

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[147] Wadsworth, J. et al., **2001**.
[148] Bonetta, L., **2001.** Scientists Race to Develop a Blood Test for VCJD, **Nature Medicine**, 7, p. 261.
[149] Hill, A. et al., **2001.** Investigation of Variant Creutzfeldt-Jakob Disease and Other Human Prion Diseases with Tonsil Biopsy Samples.
[150] Schreuder, B. et al., **1998.** Tonsillar Biopsy and PrP-sc Detection in the Preclinical Diagnosis of Scrapie, **Veterinary Records**, 142, pp. 564-568.

onset of symptoms.[151] No studies examining the accumulation of PrP-sc in the third eyelid of people have been reported.

PrP-sc has been found also in the urine of infected animals. In hamsters, the only animals tested, PrP-sc can be detected in the urine in one to two months post exposure, a time when PrP-sc is beginning to accumulate in the brain.[152] Interestingly, the PrP-sc found in urine is not infectious.[153] The testing of urine has the advantage of being minimally obtrusive to the subject. However, no other laboratory has reported successful repetition of these results.

Note: because it is not possible to identify the day at which a person becomes infected with vCJD, these tests cannot be analyzed for pre-clinical diagnosis of vCJD.

Diagnosis of CJD

Because the two diseases have distinct causes and progression it is useful to be able to distinguish CJD from vCJD. CJD patients display well characterized symptoms (bright spots) when their brains are examined by diffusion-weighted magnetic resonance imaging.[154] Most vCJD patients display no characteristic pattern when examined with the same method, providing a differential diagnosis for some patients.

CJD patients also accumulate 14-3-3 protein (a marker for neurological damage) in the cerebrospinal fluid. This protein can be detected by standard immunological methods.[155] Most vCJD patients do not accumulate 14-3-3 protein in the cerebrospinal fluid, providing a differential diagnosis for some patients. Although both of these tests are useful at diagnosing CJD in humans, the absence of either sign mitigates their use for the diagnosis of vCJD. Furthermore, each test can only detect changes after symptoms occur.

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[151] O'rourke, K. et al., **2000.** Preclinical Diagnosis of Scrapie by Immunohistochemistry of Third Eyelid Lymphoid Tissue, **The Journal of Clinical Microbiology**, 38, pp. 3254-3259.
[152] Shaked, G. et al., **2001.** A Protease Resistant PrP Isoform is Present in the Urine of Animals and Humans Affected with Prion Diseases, **The Journal of Biological Chemistry**, 276, pp. 31479-31482.
[153] Shaked, G. et al., **2001.**
[154] Mittal, S. et al., **2002.** Correlation of Diffusion-Weighted Magnetic Resonance Imagining with Neuropathology in Creutzfeldt-Jakob Disease, **The Archives of Neurology**, 59, pp. 128-134.
[155] Hsich, G. et al., **1996.** The 14-3-3 Brain Protein in Cerebrospinal Fluid as a Marker for Transmissible Spongiform Encephalopathies, **The New England Journal of Medicine**, 335, pp. 924-930.

Table 10. State of the art in TSE diagnosis

Method	Usefulness prior to appearance of symptoms	Disadvantages
Examination of Brain Tissue upon Autopsy	Accumulates 1 month after intercerebral inoculation Accumulates 3 months after ingestion of high doses (Approximately 8 months prior to onset of symptoms)	Requires death of the subject
Examination of Blood	Accumulates 2 months after inoculation after injection (9 months prior to onset of symptoms)	Of questionable use Only observable early in high-dose inoculations
Examination of Tonsils	Accumulates 10 months after natural infection (30 months prior to onset of symptoms)	Relatively invasive
Examination of Third Eyelid	Accumulates 20 months after natural infection (20 months prior to onset of symptoms)	Relatively invasive Of questionable use in humans
Examination of Urine	Accumulates 1 month after high-dose inoculation in laboratory (10 months prior to onset of symptoms)	Unconfirmed experiment Of questionable relevance to natural infections
Brain Imaging and Detection of 14-3-3 Protein	Of questionable use in pre-symptomatic patients	Only useful for diagnosis of CJD not vCJD

2B. Other Potential Diagnostic Methods

The demand for rapid and cheap tests of BSE infection in cattle has grown in the last year. On July 1, 2001, the UK mandated that all cattle over 30 months old had to be tested for BSE prior to their sale as beef.[156] Clearly, labor-intensive assays that are common today should be replaced with automated or simple diagnostic tests.

In this section, novel methods to diagnose pre-clinical subjects that will fall ill of TSEs will be discussed. Most of these methods will focus on biometric criteria of infection because of the uncertain association of PrP-sc with TSEs. Clearly, any of the novel techniques for detecting prion contamination in samples could be used for the analysis of blood, urine, tonsils, third eyelids or any other tissue that is of diagnostic value. The enhanced sensitivity of the novel assays may benefit the diagnostician by making a clear identification of PrP-sc contaminated tissues earlier than other tests.

[156] Bonetta, L., **2001**. More Sensitive Tests for BSE Needed, **Nature Medicine**, 7, p. **261**.

Diagnosis of vCJD by Imaging of the Brain's Metabolism

Neurons co-cultured in vitro with PrP-sc lose associated superoxide disumatase activity, leaving them more susceptible to oxidative damage.[157] After the loss of superoxide disumtase acivity, the ability of the neurons to metabolize glucose decreases, possibly due to mitochondrial damage.[158] Therefore, a potential method to diagnose pre-clinical vCJD patients would be to image the rate of glucose metabolism in the brain. By the injection of subjects with radiolabeled 2-fluoro-2-deoxy-D-glucose followed by positron emission tomography, the relative levels of glucose metabolism can be measured in brain tumors and their surrounding tissue.[159] Similar methods have been successfully used to image the lesions in the brains of patients afflicted with FFI and classical CJD.[160],[161] Because a drop in glucose metabolism precedes neuronal death from PrP-sc in the laboratory, this type of imaging should be able to diagnose pre-clincal patients with vCJD. Due to the high cost of a positron emission spectrometer, this type of testing should only be done on human subjects who suspect infection with the vCJD agent. A recent report demonstrates that 2-fluoro-2-deoxy-D-glucose is useful for the diagnosis of classical CJD.[162] This report did not mention attempts to diagnose vCJD with this technique.

Transcriptional Analysis of White Blood Cells

As described above, analysis of transcriptional changes in leukocytes in response to the presence of PrP-sc can be useful for the identification of infectious material in samples. A slight variation on this experiment would produce a highly-sensitive and early assay for human and animal exposure to the agent of BSE/vCJD. For this diagnostic to be

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[157] Thackray, A. et al., **2002.** Metal Imbalance and Compromised Anitoxidant Function are Early Changes in Prion Disease, **Biochemical Journal**, 362, pp. 253-258.
[158] Miele, G. et al., **2002.** Ablation of Cellular Prion Protein Expression Affects Mitochondrial Numbers and Morphology, **Biochemical and Biophysical Research Communications**, 291, pp. 373-377.
[159] Weber, W. et al., **2000.** Quantitative Assessment of Tumor Metabolism using FDG-PET Imaging, **Nuclear Medical Biology**, 27, pp. 683-687
[160] Cortelli, P. et al., **1997.** Cerebral Metabolism in Fatal Familial Insomnia, **Neurology**, 49, pp. 126-132.
[161] Matochik, J. et al., **1995.** Regional Cerebral Glucose Metabolism in Autopsy-Confirmed Creutzfeldt-Jakob Disease, **Acta Neurologica Scandinaviae**, 91, pp. 153-157.
[162] Henkel, K. et al., **2002.** Positron Emission Tomography with [(18)F]FDG in the Diagnosis of Creutzfeldt-Jakob Disease (CJD), **The Journal of Neurology**, 249, pp. 699-705.

developed, more basic data is needed on the reaction of white blood cells to infection by BSE.

In this experiment, monkeys (for the human assay) and cows (for the bovine assay) would be fed infectious material. Leukocytes would be harvested from the infected animals prior to the infection and set times after the infection (daily). Through DNA microarray analysis the levels of transcription of the genes in the leukocytes will be analyzed over the course of the disease. After it is determined which 10 genes have their transcription most altered by infection with BSE/vCJD, they are used as the basis for diagnostic tests in humans and cattle. If a subject is suspected of being infected with BSE/vCJD, a blood sample is taken and the transcription of the 10 target genes in leukocytes is analyzed by conventional methods (northern blot, mini-DNA-microarray). Since white blood cells are the first cells to interact with PrP-sc, they will probably be the first cells to show transcriptional differences, possibly prior to the accumulation of PrP-sc in the urine or brain. Note that expensive experimental animals, monkeys and cows, are only used to generate the base-line data for this test. They are not used routinely for diagnosis once the preliminary data is gathered, reducing the potential cost of this test.

Diagnosing BSE/vCJD by Changes in the Heartbeat

One of the first areas of the brain to be affected by BSE/vCJD is the solitary nucleus, an area which controls the heart beat. Researchers have found that animals infected with BSE demonstrate a characteristic heart beat arrhythmia 2 months before the onset of symptoms.[163,164] This arrhythmia can be detected by taking an electrocardiogram and recording the breathing of the subject for 5 minutes. Currently, patients with vCJD are undergoing similar tests to determine if this diagnostic works on human patients. Although much of the data is still unavailable for scientific scrutiny, this tool could potentially be useful for identifying pre-symptomatic subjects.

Diagnosis of TSEs by Changes in Metal Content in the Blood

As stated above, Prp-sc differs from PrP-sen in which metal ions it binds. This finding led researchers to examine the relative levels of metals in various tissues of mice

[163] Austin, A. et al., **1996.** Heart Rate Variability in BSE, **Veterinary Records**, 139, p. 631
[164] Franz, S., **2002.** Heartbeat Clue to Diagnosing vCJD, **Nature Medicine**, 8, p. 431.

infected with BSE.[165] The researchers found that the levels of copper in the brain and liver were markedly different between infected and uninfected animals. However, the analysis of these tissues for metal content requires the sacrifice of the animal and is no more sensitive or specific than analysis of PrP-sc content by ELISA. However, researchers also found elevated levels of manganese in the blood of infected animals between 30 and 90 days after inoculation (5 months prior to the onset of symptoms). If this finding proves to be translatable into natural infections and to mankind, an extremely easy and rapid test for BSE/vCJD infection would be to analyze manganese content in the blood by flame spectrometry. However, as the levels of manganese in the blood dropped back to normal levels after 90 days post-inoculation, this diagnostic is only useful if the exposure date is suspected.

Diagnosis of BSE by Exaggerated Response to Infection

It has long been known that patients with Alzheimer's disease are more susceptible to infection and that even minor infections produce exaggerated symptoms in these patients (such as high-fever and pronounced malaise). Researchers have found that animals infected with BSE have an exaggerated symptomatic response to infection as well.[166] Mice infected with BSE move less than uninfected littermates when challenged with a sublethal dose of bacterial lipopolysaccharide. Furthermore, 3 hours after inoculation with the bacterial antigen, mice infected with BSE show a marked drop in body temperature (2-3 deg. C lower than uninfected controls). This difference in body temperature change first presents 4 months after inoculation (roughly 7 months before other symptoms present). This experiment should be repeated with cattle infected by orally. If this finding is translatable to cattle, animals can be tested for BSE infection by injecting them with a sublethal dose of lipopolysaccharide and then measuring their core temperature 3 hours post-injection. This experiment is simple enough to be performed by unskilled workers and is extremely cheap. Due to the necessity of injecting a known

[165] Thackray, A. et al., **2002.** Metal Imbalance and Compromised Antioxidant Function Are Early Changes in Prion Disease, **The Biochemical Journal,** 362, pp. 253-258.
[166] Combrinck, M. et al., **2002.** Peripheral Infection Evokes Exaggerated Sickness Behavior in Pre-clinical Murine Prion Disease, **Neuroscience,** 112, pp. 7-11.

bacterial antigen into the subject, this test would not be an acceptable diagnostic for the testing of people.

Diagnosis of BSE by the Antibody Content in Serum

As stated in the introduction, there is no immunological response against any pathogen in BSE/vCJD infection. However, after symptoms appear, subjects with CJD, kuru or BSE possess antibodies against components of neural tissue (anti-neurofilament or anti-myelin) in their serum.[167] Blood can easily be screened for the content of these antibodies by a microfluidic ELISA. Briefly, a minute amount of blood is drawn over a surface with neural antigens immobilized. If the blood contains antibodies to these components, they will adhere to the antigen and can be detected by a secondary antibody. Although this test is only useful late in the disease, the ease and rapidity of this test allows its use in the testing of all cattle unable to walk to slaughter, "downer" cattle. Rigorous experiments need to be completed to analyze the earliest times in which these antibodies first appear in the blood, perhaps allowing this test to be used as a simple diagnostic for pre-symptomatic people and animals.

Diagnosis of TSEs by Plasmin Activity in the Blood

As stated above, plasminogen is bound and activated through the action of PrP-sc. To date, no group has reported the elevation of plasmin (the activated form of plasminogen) activity in the blood of subjects with BSE/vCJD. Clearly, an experiment needs to be performed in which the activity of serum plasmin is measured after infection with BSE in cattle. The presence of increase plasmin activity in the blood of infected subjects could be measured by the simple enzymatic assay described above.

Diagnosis of TSEs by EDRF Expression in Blood

Erythroid differentiation-related factor (EDRF) is expressed in tissues of the lymphoid lineage (blood, bone marrow and spleen). By differential display analysis, it was found that the levels of EDRF transcription in the spleen were markedly down-

[167] Tiwana, H. et al., **1999.** Autoantibodies to Brain Components and Antibodies to *Acinetobacter calcoaeticus* Are Present in Bovine Spongiform Encephalopathy, **Infection and Immunity**, 67, pp. 6591-6595.

regulated in animals that were infected with BSE.[168] Furthermore, levels of EDRF transcription were lower in whole blood of animals infected with BSE than in uninfected littermates. The drop in expression (20% drop) was first noticeable 40 days after inoculation (10 months prior to the onset of symptoms) and the decrease became more pronounced until death. There have been no reports on the levels of EDRF transcription in humans infected with vCJD. Furthermore, the expression levels of the EDRF protein have not been measured as there is currently no antibody against the protein. Once this antibody exists and a matching decrease in the expression level of the protein is found in infected animals, a quantitative microfluidic-based ELISA for EDRF protein could be performed on blood to diagnose BSE or vCJD. Since there is no protease treatment step, this assay should be more rapid and give fewer false positive results than ELISAs that detect PrP-sc in urine (which is first noticeable in a similar time frame, 30 days after infection). Furthermore, this test measures the body's response to BSE infection and not PrP-sc accumulation, which may not always be related.

[168] Miele, G. et al., **2001.** A Novel Erythroid-specific Marker of Transmissible Spongiform Encephalopathies, **Nature Medicine**, 7, pp. 361-364.

Table 11. Novel methods to diagnose TSEs in cattle and people

Method	Advantage	Disadvantage
Imaging the Brain's Metabolism	Non-invasive diagnosis Does not depend on presence of PrP-sc Uses Standard Medical Equipment Possibly allows pre-clinical diagnosis	Expensive At best a late, circa-pre-clinical diagnsosis
Transcriptional Analysis of White Blood Cells	Minimally invasive Potentially an extremely sensitive and specific diagnostic that could identify infection a few days after it occurs	More experiments need to be completed prior to the development of this assay
Change in Heartbeat	Extremely simple and cheap Diagnosis of illness 2 months prior to symptoms	Little published data available for analysis Has not be repeated by other labs No information on naturally infected animals or humans
Metal Content in Blood	Simple and cheap Diagnosis of illness 5 months prior to onset of symptoms	Only useful for a short time window in the course of disease No information on naturally infected animals or humans
Exaggerated Response to Infection	Extremely simple and cheap Diagnosis of illness 7 months prior to symptoms Works to diagnose similar disease in humans	Cannot be done in humans because depends on injection of bacterial antigens
Antibody Content in Serum	With microfluidic interface, simple and cheap Possibly late-pre-clinical diagnosis of illness	No evidence for pre-clinical appearance of autoantibodies
Plasmin Activity in Blood	Simple, cheap and rapid Possibly early-pre-clinical diagnosis of symptoms	No in vivo evidence for enhanced plasmin activity in the blood Possibly only related to PrP-sc content and not BSE/vCJD status
EDRF Expression in the Blood	Diagnosis 10 months prior to the onset of symptoms	Technically demanding (unless automated via microfluidics) Requires quantitative readout

2 C. Reccomendations

Clearly, diagnostics that are simpler, more rapid and less invasive than today's assays are required. Many of the diagnostics suggested rely on data that has not been repeated by more than one laboratory or that has not been demonstrated in mankind or naturally infected cattle. However, once experiments surface that support the initial findings, several diagnostics should be developed.

Simple assays that depend only on blood samples should be done to screen blood donors and suspect cattle (such as the plasmin assay, measurement of response to infection, serum antibody content). These assays are cost-effective enough to be used in

the many occasions where heightened biosecurity is desired. When a more rigorous or sensitive assay is demanded, either the transcriptional regulation assay or EDRF expression assay should be performed due to its use of blood products and non-reliance on the appearance of PrP-sc.

3. Therapeutics that Prevent or Treat vCJD

3A. State of the Art

All those who have been diagnosed with vCJD have died of the disease. At present, there is no therapy that is able to interrupt the course of vCJD or reverse its effects. This therapeutic gap is not due to a lack of compounds that inhibit the formation of PrP-sc in vitro. Several compounds have been found that interrupt the formation of PrP-sc or dissolve the prion aggregates, such as congo red and its analogs, trypan blue, Sirius red, various branched polyamines, porphyrins, phthalocyanines, amphotericin B and quinacrine.[169,170,171,172,173,174,175]

Some researchers have designed compounds to inhibit the action of PrP-sc specifically. Realizing that certain Prp alleles inhibit the development of scrapie even when expressed along with susceptible alleles, researchers have made non-peptide mimics of the critical region encoded by the dominant allele.[176] Others, realizing that PrP-sc folds into a β-sheet dominated structure that is essential to its function, have

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[169] Rudyk, H. et al., **2000.** Screening Congo Red and Its Analogues for Their Ability to Prevent the Formation of PrP-res in Scrapie-infected Cells, **The Journal of General Virology,** 81, pp. 115-1164.
[170] Supattapone, S. et al., **2002.** Pharmacological Approaches to Prion Disease, **Biochemical Pharmacology,** 63, pp. 1383-1388.
[171] Supattapone, S. et al., **2001.** Branched Polyamines Cure Prion-Infected Neuroblastoma Cells, **The Journal of Virology,** 75, pp. 3453-3461.
[172] Demaimay, R. et al., **2000.** Inhibition of Formation of Protease-resistant Prion Protein by Trypan Blue, Sirius Red and Other Congo Red Analogs, **The Archives of Virology Supplement,** 16, pp. 277-283.
[173] Caughey, B. et al., **1993.** Congo Red Inhibition of Scrapie Agent Replication, **The Journal of Virology,** 67, pp. 6270-6272.
[174] Caughey, W. et al., **1998.** Inhibition of Protease-resistant Prion Protein Formation by Porphyrins and Phthalocyanines, **The Proceedings of the National Academy of Sciences of the USA,** 95, pp. 12117-1212.
[175] Mange, A. et al., **2000.** Amphotericin B Inhibits the Generation of the Scrapie Isoform of the Prion Protein in Infected Cultures, **The Journal of Virology,** 74, pp. 3135-3140.
[176] Perrier, V. et al., **2000.** Mimicking Dominant Negative Inhibition of Prion Replication Through Structure-based Drug Design, **The Proceedings of the National Academy of Sciences of the USA,** 97, pp. 6073-6078.

developed peptides that bind to β-sheets and disrupt them.[177] Although both of these compounds were able to dissolve PrP-sc aggregates in vitro, reverse the formation of PrP-sc aggregates in cell lines ex vivo, and delay the onset of BSE in infected mice, none were able to reverse the course of the disease or delay its progression in mice already presenting with symptoms of BSE.

Part of the failure of these compounds is undoubtedly due to the impermeability of the blood-brain-barrier, which prevents passage of most molecules from the bloodstream into the brain. Another possible reason for failure is the possibility that the formation of PrP-sc is symptomatic but not the cause of the progression of vCJD. Undoubtedly, the main difficulty in the treatment of any neurodegenerative disease is the fact that once symptoms appear, many essential brain cells have died and reversal of the course of the disease would require re-growth of the tissue, which is currently beyond therapeutic intervention.

None of these compounds completely prevent the progression of BSE in infected mice. Experiments have not been reported that measure the infectious dose as a function of therapeutic agent administration to see if the therapeutics would have some protective effect.

3B. Strategies for the Discovery of New vCJD Therapeutics/Chemo-prophylactic Agents

Repairing the Brain

To date, the only technique that has come close to the regeneration of nervous tissue is the adoptive transfer of embryonic stem cells into sites of neurological damage. Although there are moral and political issues surrounding these efforts, the work is too promising to shelve. Only through the regeneration of neurons will patients already showing symptoms of any neurodegenerative disorder gain any improvement in the quality of life.

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[177] Soto, C., et al., **2000. Reversion of Prion Protein Conformational Changes by Synthetic β-sheet Breaker Peptides, The Lancet,** 355, pp. 192-197.

New Targets for Therapeutics

To date, all rationally designed therapeutic compounds have focused on PrP-sc itself. No effort has been made to target the possible receptors of PrP protein or other factors that interact with prions. The only known receptor for PrP-sc in the brain is the neurotrophin p75 receptor.[178] Finding compounds that modulate the activity of this receptor, either by rationally designing compounds that fit into the receptors binding site or screening for inhibitory compounds in a high-throughput screen, could potentially block the progression of vCJD.

A major effort of drug discovery companies is the search for compounds that modulate apoptosis. Due to the fact that the neurons in vCJD patients die via the apoptotic pathway, anti-apoptotic drugs could prevent tissue damage. Because the proper progression of apoptosis prevents auto-immune disorders and cancer, this drug would have to sequestered in the brain. This sequestration could be accomplished by designing the drug to be impermeable to the blood-brain-barrier and injection of the compound intercerebrally to prevent escape of the compound from the brain. In fact, an apoptosis inhibitor, CEP-1347, has entered human clinical trials for the treatment of neurodegenerative diseases unrelated to cVJD (although this compound is permeable to the blood brain barrier and consequently may have unintended side-effects).[179]

As discussed in the introduction, there is a marked difference between the levels of Prp mRNA and levels of PrP protein in many neuronal cells. The mechanism of this post-transcriptional down-regulation is unclear. If the factor that causes this downregulation is found, and it specifically acts on the Prp mRNA, it would be an excellent target for therapeutic intervention as cells that do not express PrP protein are immune to prion diseases. The identification of this factor should be a major research effort. All-trans retinoic acid has been demonstrated to downregulate the transcription and translation of PrP. Although its effects are not specific to the factor that executes the

[178] Della-Bianca, V. et al., **2001.**

[179] Maroney, A. et al., **2001.** CEP-1347 (KT7515), a Semisythetic Inhibitor of the Mixed Lineage Kinase Family, **The Journal of Biological Chemistry**, 276, pp. 25302-25308.

post-transcriptional regulation of Prp, retinoic acids should be pursued as possible therapeutic agents in the progression of prion disease.[180]

For most compounds that have anti-PrP-sc activity ex vivo, such as congo red, the protein through which they work remains obscure. The proteins that bind congo red and its analogues could become the target for the next generation compound screen for therapeutics that disrupt the progression of prion diseases. To find these proteins, a radio-affinity fishing experiment must be conducted. Congo red would be made radioactive and modified with linkers that will covalently attach congo red to any protein it binds to. The identification of its receptor is done by biochemical analysis of the resulting congo-red-receptor adduct that is identifiable through its radioactive label.

PrP Protein as a Therapeutic

Efforts to mimic parts of the PrP protein have centered on moieties that disrupt the aggregation or folding of PrP-sc. However, it may be prudent to use molecules that mimic the portions of PrP that participate in the folding or aggregation. These molecules would resemble parts of the PrP protein essential for binding to PrP-sc (most likely in the region that encompases amino acids 168, 172, 215 and 219[181]). However, since these molecules only resemble the portion that binds to PrP-sc, they cannot participate in the transmutation of other proteins and may act as a cap to dead-end a growing aggregate. This strategy could prevent the self-replication of prions in the body.

RNAi

Cells that do not express PrPsc are immune to prion disease, and mice lacking the Prp gene seem normal (although there is obviously some essential function of the PrP protein otherwise it wouldn't be as conserved as it is through evolution). The use of viruses to knock out genes in an adult animal is untenable due to the haphazard and non-specific genomic disruptions caused by such viruses. Recently, a phenomenon first discovered in plants, led to a new tool in the regulation of expression in adult animals. It was observed that if plants were injected with a double-stranded molecule of RNA

[180] Rybner, C. et al., **2002.** All-trans Retinoic Acid Down-regulates Prion Protein Expression Independently of Granulocyte Maturation, **Leukemia**, 16, pp. 940-948.
[181] Perrier, V. et al, **2000.**

(inhibitory RNA or RNAi) homologous to a stretch in a gene, that gene would be shut off in the entire organism.[182] This phenomenon has been observed in animals (such as roundworms) and has a more limited effect in mammalian cells. RNAs are not taken up by mammalian cells and special modifications of the RNAi would have to be made to allow entry into the cell where it would down-regulate the Prp gene. Treating the RNA with poly-lysine would mask the negative charges on the molecule and allow entry into the cell via membrane diffusion. Alternatively, the RNAi could be modified with a cell penetrating peptide (such as antennapedia) to get it to its site of activity. By intercerebrally injecting people infected with vCJD with modified-RNAi specific for the Prp gene, it would be possible to shut off all of the prion production in the brain, preventing the progression of prion diseases. This treatment may only need to be temporary as clearance of prion aggregates from the body (via the urine) may occur as no new aggregates are made.

High-throughput Screen for Inhibitors of the vCJD Agent

The majority of the therapeutic compounds discovered in the last decade were not designed, but found in high-throughput assays that can screen thousands of compounds a day. Similar screens could be designed for anti-vCJD therapeutics, enabling the discovery of unexpected classes of drugs to treat the disease.

In this assay, neuroblastoma cells, which are susceptible to killing by PrP-sc, are grown in a thousand-well tissue culture plate. These cells are genetically modified to synthesize acquorin (a luminescent protein) when apoptosis is triggered, and thereby emit light. PrP-sc and a compound from a drug library is added to each well. Possible lead compounds will be found in wells that are not emitting light due to the absence of apoptosis in those cells. This assay would be relatively easy to develop but the lack of a market for these drugs (due to the rarity of the disease), combined with the cost of testing lead compounds, will prevent this assay from being realized.

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[182] Vaucheret, H. et al., **2001.** Post-transcriptional Gene Silencing in Plants, **The Journal of Cell Science**, 114, pp. 3083-3091.

Enhancing the Immune Response to PrP-sc

There is no immune response against the invasion of prions into the host and poor immuno-reactivity of the prion protein prevents vaccines from being made against it. However, recent experiments have demonstrated that the immune system can be primed to fight-off vCJD. Mice transfected with a gene encoding an anti-PrP-protein antibody did not develop BSE after experimental challenge (further supporting the theory that PrP-sc causes BSE).[183] Unexpectedly, these mice did not develop autoimmune disease toward neuronal cells, possibly due to the immunological privilege of the brain. Similar techniques could be used to lend adoptive immunity to those patients potentially exposed to the vCJD agent. Human B-cells (which produce antibodies) can be transfected to allow expression of a humanized version of the murine anti-prion antibody. These cells would then be injected into the patient to allow expression of the antibody in the serum. Many B-cell lines would have to be produced to match the potential immunological allotypes of a patient to prevent the patient's immune system from destroying the adopted cells. This technique is superior to the simple injection of antibodies into a patient because the adopted B cells will proliferate, providing long-lasting immunity with one treatment.

Non-specifically stimulating the immune system has shown some promise in mouse models of disease. The injection of oligonucleotides consisting solely of repeating cytosine-guanine pairs stimulates leukocytes. Such treatment has been demonstrated to slow the onset and progression of BSE in mice injected with prions.[184] Importantly, this treatment works even when administered several days after prion exposure.

Preventing Leukocyte Uptake of PrP-sc

A recent epidemiological study has found that fewer vCJD patients have the DQ7 allele of the major histocompatibility type II locus (which encodes a receptor on professional antigen presenting cells such as leukocytes) than the general population.[185] The authors hypothesize that this allele somehow confers a modicum of protection against vCJD by guiding an immunological response. The authors make no mention of

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[183] Heppner, et al., **2001.** Prevention of Scrapie Pathogenesis by Transgenic Expression of Anti-Prion Protein Antibodies, **Science,** 294, pp. 178-182.
[184] Sethi, S. et al., **2002.** Postexposure Prophylaxis against Prion Disease with a Stimulator of Innate Immunity, **The Lancet,** 20, pp. 229-230.
[185] Jackson, G. et al., **2001.** HLA-DQ7 Anitgen and Resistance to Variant CJD, **Nature,** 414, p. 269

why there is no immunological response in animals if such a response is important in defense against the disease.

What the authors fail to mention, however, is that there is an equally significant *increase* in the number of vCJD patients who have the DQ6 allele over the uninfected population. Since leukocytes are thought to carry the protein, it could be that the DQ6 allele acts as a receptor for the binding of prions to the cell. Major histocompatibility proteins bind their targets via a peptide binding cleft in their extra-cellular domain, the specificity of which is known for each allele. The addition of excess peptides that bind to the DQ6 allele could potentially block uptake of PrP-sc by white blood cells and prevent the entry of prions into the brain.

Unfortunately, the major histocompatibility type II alleles of cattle are largely uncharacterized, preventing a similar study with the larger pool of BSE infected cattle. The major histocompatibility type II alleles of mice are completely characterized however, and the theories above can either be proved or disproved by the identification of murine alleles that either inhibit or assist pathogenesis.

Leukodepletion

Several studies have pointed to the importance of leukocytes in the progression of BSE. However, attempts to block the function or maturation of circulating white blood cells only marginally slow the onset of prion diseases.[186,187,188,189] Some have cited that this failure in prevention of BSE is indicative of the fact that the wrong type of leukocytes are being targeted by these therapies and that other classes of white blood cells are responsible for the transport of prions from the gut to the lymph nodes.[190,191]

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[186] Mabbott, N. et al., **2000.** Temporary Inactivation of Follicular Dendritic Cells Delays Neuroinvasion of Scrapie, **Nature Medicine,** 6, pp. 719-720.
[187] Manuelidis, L. et al., **2000.** Folicular Dendritic Cells and Dissemination of Creutzfeldt-Jakob Disease, **The Journal of Virology**, 74, pp. 8614-8622.
[188] Mabbott, N. et al., **2002.** Temporary Blockade of the Tumor Necrosis Factor Receptor Singalling Pathway Impedes the Spread of Scrapie to the Brain, **The Journal of Virology,** 76, pp. 5131-5139.
[189] Cardone, F. and Pocchiari, M., **2001.** A Role for Complement in Transmissible Spongiform Encephalopathies, **Nature Medicine**, 7, pp. 410-411.
[190] Oldstone, M. et al., **2002.** Lymphotoxin-a- and Lymphotoxin-b-deficient Mice Differ in Susceptibility to Scrapie: Evidence Against Dendritic Cell Involvement in Neuroinvasion, **The Journal of Virology,** 76, pp. 4357-4363.
[191] Prinz, M. et al., **2002.** Lymph Nodal Prion Replication and Neuroinvasion in Mice Devoid of Follicular Dendritic Cells, **The Proceedings of the National Academy of Sciences of the USA,** 99, pp.919-924.

Although the particular kind of leukocytes involved in the transport of prions from the gut is unclear, the removal of all white blood cells from circulation after infection with PrP-sc would undoubtedly benefit the patient. Blood products are routinely stripped of their leukocytes prior to their administration to patients in a highly automated process. If a subject is known to have been exposed to prions, his/her blood could be filtered in an analogous way to remove all leukocytes (and probably the vast majority of circulating prions). Due to the immunological impact (and cost) of such a procedure, it must only be performed if it was clear that the patient was exposed to a lethal dose of prions.

Table 12. Possible future treatment options for vCJD

Therapeutic Regime	Advantage
Repairing the Brain	Possibly the only way (albeit a morally dubious one) to reverse the damage after symptoms appear
New Targets	Circumvents failures in anti-PrP drugs by targeting other players in the pathway The blockade of the PrP receptor may prevent PrP-sc toxicity The modulation of apoptosis may prevent neural death in the presence of PrP-sc The reduction of PrP-sen production through its regulator could prevent or reverse the course of the disease The discovery of the targets of current in vitro therapeutics may lead to new classes of drugs that work in vivo
PrP protein	"Capping" PrP therapeutics may prevent aggregation and replication of PrP-sc
RNAi	Modified RNAi could be used to block expression of PrP-sc preventing accumulation and replication of prions
Novel High-throughput Screen	Compatible with current drug discovery regimes Useful in the discovery of unpredicted classes of therapeutics
Antioxidants	May prevent damage to neurons by vCJD
Enhancing the immune response	Adoptive transfer of B-cells could prevent development of vCJD, not cure it
Preventing Leukocyte Uptake of PrP-sc	Treatment with drugs that block the binding cleft of the DQ6 allele could prevent uptake of prions
Leukodepletion	Removal of all circulating white blood cells (the first reservoir of PrP-sc) should drastically reduce pathogen titers

3C. Recommendations

Most of the therapeutic regimes described above require substantial further basic research. The use of RNAi as a therapeutic is largely unexplored and funding should be provided to support basic research on developing RNAi as a deliverable drug. The therapeutics described above could all be tested in animal models of scrapie or BSE in the near future to determine if pursuit of these compounds or methods for use in mankind is warranted. To cover the expenses of clinical trials and animal testing, large pharmaceutical companies claim to require a new drug to sell $1 billion a year to cover expenses. Although there are certain provisions that make the discovery of therapeutics

for rare diseases more attractive (orphan drug status), more needs to be done. This support could come in the form of public funding or relaxed clinical trials to determine the safety of drugs that benefit the terminally ill.

4. Methods to Prevent the Spread of BSE and scrapie

4A. State of the Art

The current method to prevent the spread of BSE is to ban the feeding of cattle with feed derived from other animals. Since the only suspected mode of transmission of BSE is through the ingestion of contaminated foodstuffs, this measure will probably lead to the eradication of the disease within the next few years. Although currently unknown, it is possible for cattle to develop "sporadic" BSE, which may enable prions to enter the food supply, albeit in small amounts.

The method of transmission of scrapie is unknown and it will continue to be a disease endemic to most areas. Therefore, prions and the potential for natural prion contamination will continue to persist unless more drastic methods are pursued.

4B. Recommendations

Currently, vaccination against BSE and scrapie is impossible. Future methods (such as the adoptive transfer of B cells) are impractical for the scale and cost margins of animal agriculture. However, immunity to prion diseases can be gained via genetic resistance. Several lines of sheep are naturally resistant to scrapie due to a polymorphism in the Prp gene.[192,193] Similar polymorphisms are found in mankind, lending natural resistance to CJD.[194] Although breeds of cattle are more resistant to BSE than others, no breed has been found that is completely immune to BSE.

To eliminate scrapie from the industrialized world, a government-supported, selective breeding program should be adopted. Sheep should be bred with scrapie resistant lines until the entire sheep population has only the Prp alleles that confer

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[192] Hunter, N. et al., **1997.** Association Between Natural Scraie and PrP Genotype in a Flock of Suffolk Sheep in Scotland, **Veterinary Records**, 140, pp. 59-63.
[193] Ikeda, T. et al., **1995.** Amino Acid Polymorphisms of PrP with Regerence to Onset of Scrapie in Suffolk and Corriedale Sheep in Japan, **The Journal of General Virology**, 76, pp. 2577-2581.
[194] Shibuya, S. et al., **1998.** Protective Prion Protein Polymorphisms against Sporadic Creutzfeldt-Jakob Disease, **The Lancet**, 51, p. 419.

resistance to scrapie. The resistant alleles can be followed through the breeding program by conventional techniques such as southern blotting or high-stringency polymerase chain reaction. Alternatively, homozygosity for the resistant alleles can be assured by repeated back-crossing to homozygous parents, limiting the amount of molecular biology required but decreasing the genetic diversity of the population.

Because cattle have no innate immunity in their population, lines of resistant cattle must be made through genetic engineering. This can be accomplished by creating cattle with the Prp gene from resistant sheep. Alternatively, the endogenous bovine gene could be mutated to recapitulate the resistant polymorphisms available in the sheep or human population. Several genetically altered cattle lines should be developed to facilitate the selective breeding program and maintain genetic diversity. Resistant cattle should be created in each strain of cattle to maintain desirable characteristics of that breed.

Through the elimination of the primary reservoir of prion diseases by genetic engineering and selective breeding, prion diseases could be removed from the list of threats to agriculture and the food supply.

5. Summary of Recommendations

Table 13. Summary of recommendations

Recommendation
Integrate current diagnostic tests with microfluidics and precipitation steps to improve the sensitivity and ease of testing
Develop assays that identify prion infectivity by metal content, plasminogen binding or effects on cultured cells
Develop reagents (antibodies or aptamers) that bind selectively to PrP-sc in the presence of PrP-sen
Develop diagnostics to identify pre-symptomatic vCJD/BSE though non-invasive measurements (blood sampling, temperature monitoring)
Develop novel therapeutics for vCJD by targeting other molecules involved in the processing of PrP, identifying the receptors for current therapeutics that function in vitro, the facilitation of high-throughput drug discovery, modulating host immunity or developing RNAi technology
Engineer and select for resistant lines of sheep and cattle to become the mainstay of agriculture in the industrialized world

FUTURE STUDIES

To better develop methods of detection and prevention of prions and their associated diseases, more research needs to be completed. In addition to the experiments outlined above, some basic work needs to be done to understand the mechanisms of prion pathogenicity.

1. Blood Safety

The FDA has recommended that all blood banks in the US stop accepting donations from people who have spent significant amounts of time in the European Union, due to concerns that blood may be able to transmit vCJD.[195] To date, only one report suggests that blood may be infectious, and the data from this report has not been released for scientific scrutiny.[196] Whole blood was transfused in this experiment, even though transfusions rarely use whole blood today. Furthermore, the transmission of scrapie was followed. Scrapie spreads by an unknown mechanisms and it is possible that the few sheep that developed scrapie developed it for reasons unrelated to the blood transfusion.

To ascertain the real threat that vCJD poses to the blood supply, carefully controlled experiments that mimic the human blood supply must be undertaken. To do this, blood from cattle with BSE should be leukodepleted and then transfused into many uninfected cattle. Cattle should be used because the method of spread of BSE is better characterized. Leukodepleted blood should be used because that is primarily what is transfused in the industrialized world. This experiment is urgent because our blood supply is stretched dangerously thin by the refusal of thousands of healthy and willing donors.

2. Identity of PrP-sc Transporting Lymphocytes

As stated above, the scientific consensus is that leukocytes carry PrP-sc from the blood to the lymphoreticular system but there is disagreement as to the identity of the lymphocytes. The successful identification of the proper lymphocyte lineage responsible

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[195] Casagrande, R. **2001.** Bad Blood Between the FDA and Europe, **The Scientist**, 15, p. 43

[196] Houston, F., et al. **2000.** Transmission of BSE by Blood Transfusion in Sheep, **The Lancet**, 16, pp. 999-1000.

for the carriage of PrP-sc from the gut would enable better diagnostics and potential chemo-prophylaxis in the wake of a known exposure to PrP-sc. Experiments need to be completed that survey every lymphocyte type in a mouse injected with PrP-sc for prion content. Although this lymphocyte subtype has not been investigated for its role in prion pathogenicity, intenstinal intraepithelial leukocytes are a likely candidate for prion traffic from the gut to the lymphatic system due to their location in the peyer's patches in the gut.

3. Homeland Security

Although the recommendations contained in this report will help improve the response of the agricultural and medical communities to prion diseases, more needs to be done. The recommendations issued by this report will be most useful after a study in which the likely routes of prion entry into the US (either in agriculture of the US or its people) to better guide the deployment of diagnostic tests. A comprehensive study should be undertaken that would compare the likely modes of possible prion contamination of food, animals and blood either through natural or intentional means.